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The role of inactive MTMR phosphatases in mammalian cells

Úloha inaktivních fosfatáz MTMR rodiny v savčích buňkách

Diplomová práce

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Prohlášení

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Nikola Sixtová

Poděkování

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Abstrakt

Proměnlivá kompozice buněčných membrán má vliv na buněčné procesy, jakými jsou například endosomální transport, autofagie či buněčná signalizace. Na tvorbě membránové identity se významně podílí deriváty fosfatidylinositolu. Tyto deriváty jsou specificky distribuovány napříč buněčnými membránami a jejich konverze je úzce regulována souhrou příslušných lipidových kináz a fosfatáz. Myotubulariny (MTMR) tvoří rodinu fosfatáz, jež jako substráty rozpoznávají fosfatidylinositol 3-fosfát a fosfatidylinositol 3,5-bisfosfát, které následně defosforylují na pozici 3 inositolového kruhu. Podobně jako jejich substráty, i MTMR fosfatázy ovlivňují různé buněčné procesy, jako například endosomální transport či autofágii. Mutace v MTMR proteinech vedou k deregulaci buněčných procesů a sekundárně k projevům vážných patologií. Mezi nejprostudovanější patří zejména X-vázaná centronukleární myopatie a Charcot-Marie-Tooth syndrom, dvě dědičná onemocnění způsobená mutacemi v genech pro *MTM1* a *MTMR2*. Mezi specifické znaky MTMR rodiny patří přítomnost katalyticky inaktivních členů. Inaktivní MTMR proteiny regulují proteinovou stabilitu, aktivitu či buněčnou lokalizaci aktivních partnerů. MTMR10 a MTMR12 jsou dvě inaktivní fosfatázy, jež přímo interagují s aktivními MTM1 a MTMR2, čímž se řadí mezi jejich potenciální regulátory. Cílem této práce bylo prozkoumat vnitrobuněčnou lokalizaci a chování MTMR10 a MTMR12 proteinů za různých podmínek v kultivovaných lidských buňkách. V našich výsledcích ukazujeme, že uměle transfekované MTMR10 a MTMR12 proteiny jsou v buňkách distribuovány převážně v cytoplasmě a na plasmatické membráně. Společná exprese MTMR12 s aktivními partnery MTM1 a MTMR2 vede k jejich silné kolokalizaci, aniž by byla pozměněna původní lokalizace aktivních členů. Naopak se zdá, že MTMR2 částečně přivádí MTMR12 k membráně Golgiho aparátu. Také ukazujeme, že námi vybrané posttranslační modifikace MTMR12 proteinu nemají vliv na jeho lokalizaci. Při indukci autofagie pomocí hladovění dochází k relokalizaci MTMR12 do nedefinovaných struktur. Indukce autofagie pomocí hladovění částečně narušuje kolokalizaci mezi MTMR12 a MTM1 i MTMR2.

Abstract

Variable composition of the cellular membranes influences many cellular events such as endosomal transport, autophagy or cellular signalling. The membrane identity is significantly determined by the specific distribution of phosphoinositide derivatives. These derivatives are specifically distributed among cellular membranes and they are tightly regulated by the interplay of corresponding lipid kinases and phosphatases. Myotubularins (MTMRs) form a family of phosphatases dephosphorylating phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate at the 3rd position of the inositol ring. Similarly to their substrates, MTMRs are involved in various cellular events such as endosomal transport or autophagy. Mutations in MTMR proteins lead to dysregulation of the cellular events and manifestation of severe pathologies. Among the most studied are two hereditary diseases, X-linked myotubular myopathy and Charcot-Marie-Tooth syndrome, caused by mutations in *MTM1* and *MTMR2* genes, respectively. One of the specific features of the MTMR family is the presence of catalytically inactive members. These members were found to regulate protein stability, activity and localization of their active partners. MTMR10 and MTMR12 are two inactive members of the MTMR family, directly interacting with the active MTM1 and MTMR2, thus representing their potential regulators. Our aim was to study the intracellular localization and behaviour of MTMR10 and MTMR12 proteins under various conditions in cultured human cells. We show in our results that overexpressed MTMR10 and MTMR12 are distributed through the cell cytoplasm and at the PM. Co-expression of MTMR12 with the active partners MTM1 and MTMR2 leads to strong co-localization without altered localization of the active members. Interestingly, MTMR2 appears to partially recruit MTMR12 to the membrane of the Golgi apparatus. We show that these localizations are not dependent on selected MTMR12 posttranslational modifications. Additionally, we observe MTMR12 re-localization into undefined structures upon stimulation of starvation-induced autophagy. Induction of starvation appears to partially impair the co-localization between MTMR12 and MTM1 or MTMR2.

Abbreviations:

3-PAP	3-phosphatase adaptor protein
aa	amino acid
Akt	protein kinase B
Ala	alanine
AMP	ampicilin
APPL-1	adaptor protein phosphotyrosine 1
Arg	arginine
ATG12	autophagy related 12
ATG16	autophagy related 16
ATG12-5-16L	autophagy related 12-5-16L
BIN1	bridging interactor 1
CC	coiled-coil
cDM1	congenital myotonic dystrophy
CEP55	centrosomal protein 55
CMT	Charcot-Marie-Tooth
COS	CV-1, SV40
Cys	cysteine
DAPI	4',6-diamidino-phenylindole
DENN	differentially expressed in neoplastic versus normal cells
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DSP	dual-specificity phosphatase
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FYVE	Fab1, YOTB, Vac1, EEA1
GA	Golgi apparatus
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GRAM	Glucosyltransferase, Rab-like GTPase activators and myotubularin
HBSS	Hank's buffered salt solution
HeLa	Henrietta Lacks
HEK293	human embryonic kidney 293 cells
hVps5/hVPS34	human vacuolar protein sorting 5/ human vacuolar protein sorting 34
IC	intermediate compartment
Ins(1,3)P_2	inositol(1,3)-bisphosphate
KO	knock-out
LAMP1	lysosomal-associated membrane protein 1
LAS	Leica Application Suite
LB	lysogeny broth
LC3B	light chain 3B
Lys	lysine
MAPK/Erk1	mitogen-activated protein kinase/ extracellular signal-regulated kinase 1

MSB	microtubule stabilizing buffer
MTM	myotubular myopathy
MTM1	myotubularin 1
MTMR	myotubularin-related
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NF-L	neurofilament-light protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDZ	PSD-95, a post-synaptic density protein, Disc-large, a drosophila tumour suppressor and ZO-1, a tight junction protein
PEI	polyethylenimine
Pfa	paraformaldehyde
PH	plecstrin homology
PH-GRAM	plecstrin homology, Glucosyltransferase, Rab-like GTPase activators and myotubularins
PI	phosphoinositide
PI3K	phosphoinositide 3-kinase
PLK1	polo-like kinase 1
PM	plasmatic membrane
PNS	peripheral nervous system
PtdIns	phosphatidylinositol
PtdIns3P	phosphatidylinositol 3-phosphate
PtdIns4P	phosphatidylinositol 4-phosphate
PtdIns5P	phosphatidylinositol 5-phosphate
PtdIns(3,4)P_2	phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,5)P_2	phosphatidylinositol (3,4)-bisphosphate
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
PtdIns(3,5)P_2	phosphatidylinositol (3,5)-bisphosphate
PtdIns(3,4,5)P_2	phosphatidylinositol (3,4,5)-trisphosphate
PTP	protein-tyrosine phosphatase
PX	phox homology
RAB 5	RAS-associated binding5
RAB 7	RAS-associated binding7
RAB 11	RAS- associated binding 11
RAB 21	RAS-associated binging 21
RAC 1	Rat sarcoma 1
RAC 21	Rat sarcoma 21
RPE-1	retinal pigment epithelial-1
RME-8	receptor-mediated endocytosis-8
Sbf	SET-binding factor
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
Ser	serine
SHS	SH-SY5Y
SNARE	soluble NSF Attachment Protein Receptor
SID	SET-interaction domain
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TBST	Tris buffered saline with Tween

TNF	tumour necrosis factor
Tyr	tyrosine
VAMP8	vesicle associated membrane protein 8
WB	Western blot
WHAMM	WASP homolog-associated protein with actin, membranes and microtubules
WT	wild type
Wnt	wingless/Int-1
XLMTM	X-linked myotubular myopathy

1. Theoretical background

Cellular membranes are heterogeneous lipidic structures which form natural boundaries between distinct environments, namely the extracellular, intracellular and inner-organellic space. Their role in interconnection of these environments is, however, no less important. Lipidic molecules of the cellular membranes may serve either as components of signalling pathways or as mediators of cellular processes such as cell division, proliferation, endosomal transport or autophagy. Among these kinds of lipids are derivatives of phosphatidylinositol (PtdIns) called phosphoinositides (PIs) (De Craene et al. 2017).

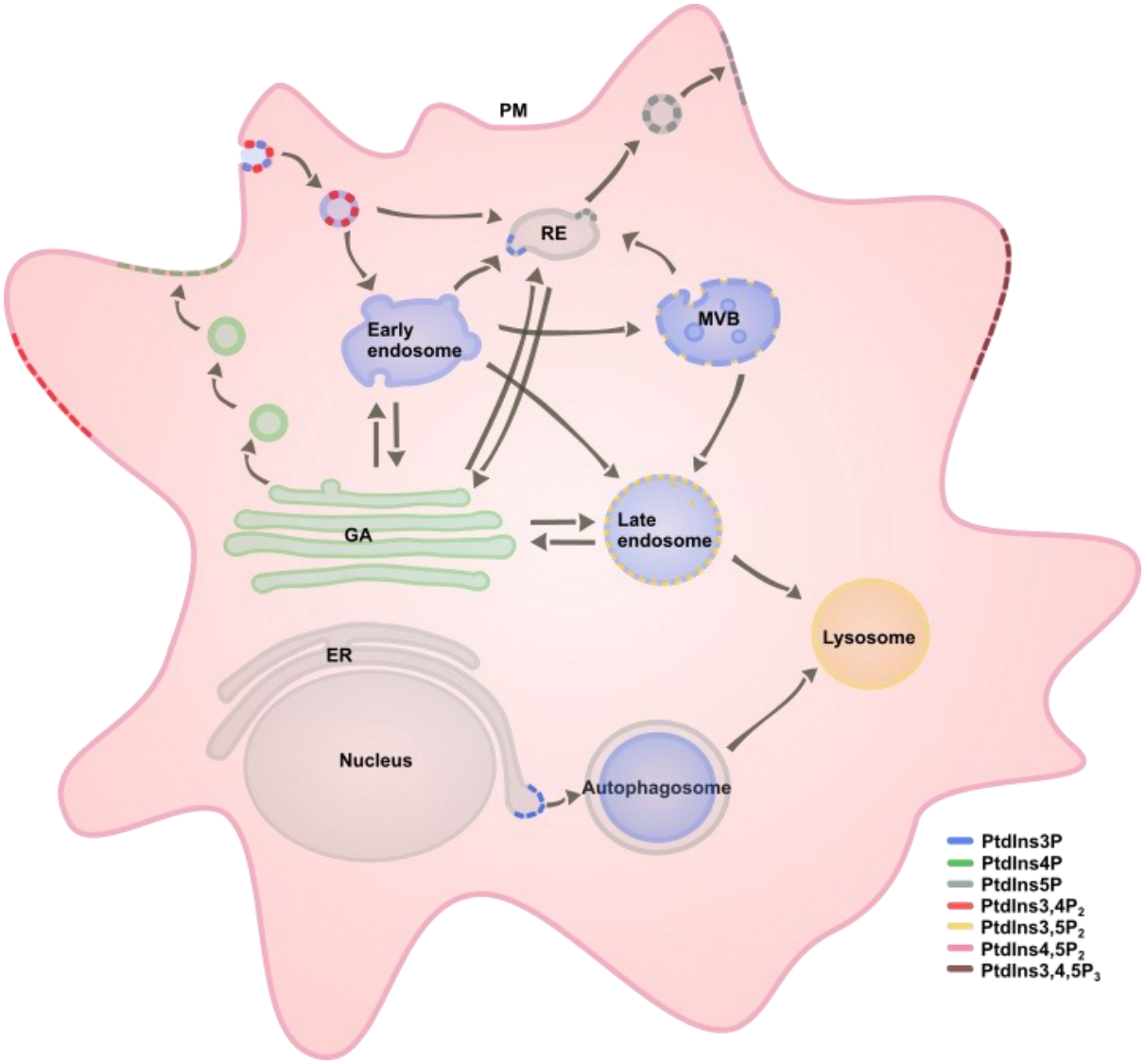


Figure 1: ***Distribution of phosphoinositide species across the cellular membranes.*** (Picture was inspired by (Hnia et al. 2012; Choy et al. 2017 and Wallroth and Haucke 2018))

PtdIns is a lipidic molecule which has its inositol polar group exposed into the cell cytosol. Approximately 80% of PtdIns molecules are present in their unphosphorylated form in the membranes of the endoplasmic reticulum (ER) or the plasmatic membrane (PM) (Dickson and Hille 2019). Due to its steric properties, only the 3rd, 4th and 5th position of the inositol ring can be phosphorylated, yet in combinations giving rise to seven derivatives - PIs. Individual PIs show distinct distribution across the cellular membranes. At the same time, the presence of each derivative is dominant in particular membranes (**Fig. 1**). The unique pattern of PIs distribution helps to create the membrane identity (Viaud et al. 2016). Two of the seven PIs species are more dominantly present in the cell, namely PtdIns(4,5)P_2 and PtdIns4P. Both derivatives are dominantly found in the PM, PtdIns4P is additionally enriched in the membranes of the Golgi apparatus (GA). Even though other derivatives represent only small portion of all PIs, their presence in the cell is still essential. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were mainly observed in the PM; PtdIns3P was characterized mainly in membranes of the early endosomes (EEs) and forming autophagosomes; PtdIns(3,5)P_2 was predominantly found in membranes of late endosomes (LEs) and the lysosomes; the last identified - PtdIns5P -, still yet poorly understood, has been observed in various endosomal compartments, in the PM and in the nuclear envelope (Viaud et al. 2016; Dickson and Hille 2019).

PIs mediate various cellular responses either by acting as second messengers, or by the recruitment of effector proteins containing respective PIs recognition domains. The first identified PI recognition domain was the pleckstrin homology (PH) domain (Harlan et al. 1994). Although the PH domain was initially shown to preferentially bind PtdIns(4,5)P_2, other variants of the PH domain were later described to bind other PIs with different affinities (Harlan et al. 1994; Lemmon 2003). Up to nowadays, several other domains, such as FYVE (Fab-1, YGL023, Vps27 and EEA1) or Phox homology (PX) domains, have been shown to bind PIs with various affinities and specificities (Gaullier et al. 1998; Xu et al. 2001; Balla 2013; Hammond a Balla 2015). The combination of unique membrane PIs composition and selective binding of the effector proteins facilitates the coordinate formation of particular protein complexes in space and time (Choy et al. 2017). It leads to precise organization and localization of individual steps of subsequent events such as endosomal transport, autophagy and others (Hnia et al. 2012). This effective mediation requires PIs to be tightly regulated in space and time.

PIs are being regulated in two ways: i) Levels of the PIs on the cellular membranes are regulated by phospholipase C. Phospholipase C hydrolyses the polar group of membrane bound

PtdIns(4,5)P_2 to generate its soluble form. The generated metabolites can either be used as building blocks for the synthesis of the future PtdIns or as second messengers in signalling pathways (Dickson and Hille 2019). ii) The particular form of PIs is achieved by an interplay between numerous kinases and phosphatases (**Fig. 2**). These enzymes work in a coordinated way to regulate PIs dynamic interconversion. While kinases modify PIs molecules by adding a phosphate group on the 3rd, 4th or 5th position of their inositol ring, phosphatases have the opposite role and removes these phosphates (Balla 2013; Dickson and Hille 2019).

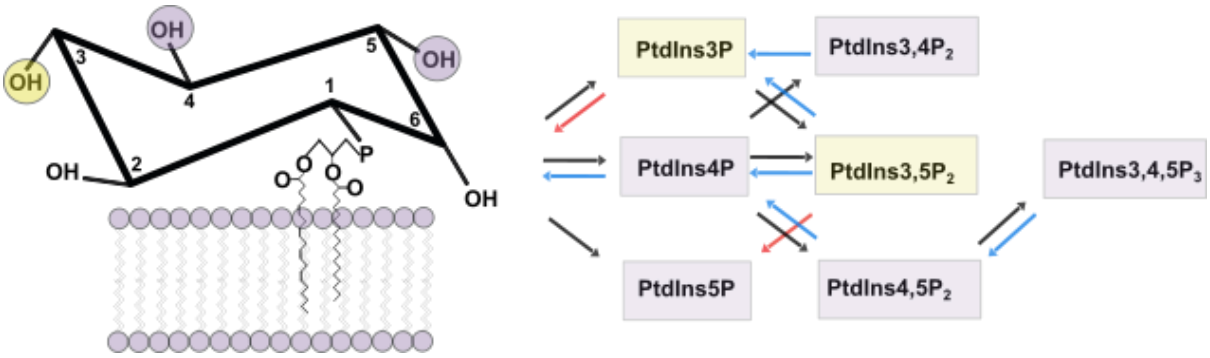


Figure 2: ***Conversion between phosphoinositide species.*** PtdIns molecule (on the left) can be phosphorylated on the 3rd, 4th and 5th position (circled) of the inositol ring. PtdIns and its derivatives are interconverted by interplay of kinases (black arrows) and phosphatases (blue arrows). Action of MTMR phosphatases is drawn with red arrows. (Picture was inspired by (Wallroth and Haucke 2018; Jean and Kiger 2012.))

Myotubularins (MTMRs) form a family of phosphatases that regulate the form of PIs by removing the phosphate group from the 3rd position of the inositol ring of PtdIns3P and PtdIns3,5P_2 substrates. Through this function, they give rise to PtdIns and PtdIns5P, respectively, change the membrane properties and influence various cellular events such as endosomal transport and autophagy (Robinson and Dixon 2006; Hnia et al. 2012).

1.1. Myotubularin family of phosphatases

MTMRs represent a large family of proteins, highly conserved through evolution from yeasts to human (Laporte et al. 1998; Lecompte et al. 2008). Based on the presence of the structural motive C(X)$_5$R in the catalytic domain, the family was classified as a specific subgroup of protein tyrosin phosphatases (PTPs) with dual function – dual-specific protein phosphatases (DSPs). In contrast to other PTPs/DSPs, MTMRs do not recognize phosphorylated amino acids (aa) but specific derivatives of PtdIns lipidic molecules PtdIns3P and PtdIns3,5P2 as substrates (Alonso et al. 2004). The founder member of the family was discovered in its mutated form in 1996 as the main cause of the X-linked myotubular myopathy (XLMTM), from which it

adopted its name “myotubularin” (MTM1) (Laporte et al. 1996). In the following years, up till now, 13 other homologues were described and named myotubularin-related proteins (MTMRs 1-13), shortly “myotubularins” (Raess et al. 2017). Additionally, the protein hJUMPY is being described in the literature as MTMR14 due to the presence of the catalytic consensus $C(X)_5R$ and the substrate specificity characteristic for other MTMRs. However, based on phylogenetical studies and the protein domain organization, hJUMPY/MTMR14 is being recognized as a distant member of the MTMR family (Tosch et al. 2006; Amoasii et al. 2013).

1.1.1. Active or dead?

Since their discovery, MTMRs have been classified as PTP enzymes based on the presence of a catalytic sequence “$C(X)_5R$” in their PTP domain, characteristic for all the members. From the yet known 14 MTMRs and 1 related protein MTMR14/hJUMPY, only 9 share the whole structural motive unaltered (MTM1-MTMR4; MTMR6-MTMR8; MTMR14) and have the ability to dephosphorylate their substrates. Remaining 6 members contain a mutation in this sequence and therefore lack the phosphatase activity (MTMR5; MTMR9-MTMR13). MTMRs enzymatic potential thus divides MTMRs into two main groups of catalytic active and “dead” phosphatases, respectively (**Fig. 3**). The presence of enzymatically inactive phosphatases is an emerging feature among the several groups of phosphatases, emphasizing their biological importance despite the fact that still not much is known about their function (Reiterer et al. 2020). Their close co-evolution with the active members suggests their importance in nature (Lecompte et al. 2008). Through the time, most of the dead MTMRs were observed to form specific hetero-dimer interactions with their active partners and regulate their cellular localization and function. This regulatory potential will be further discussed below.

1.1.2. Myotubularin protein domains

To understand the way how MTMRs work, the knowledge of their protein domain structure is essential. MTMRs are generally multidomain proteins that share the central core composed of two main domains (Raess et al. 2017). The first one is the catalytic PTP domain, which is responsible for the enzymatic activity and stands for MTMR classification as PTP/DSP proteins (Alonso et al. 2004). The second one, PH-GRAM domain, is essential in mediating interactions between MTMRs and membranes (Doerks et al. 2000; Berger et al. 2003; Tsujita et al. 2004; Choudhury et al. 2006). Outside of the catalytic core, all MTMRs, except for MTMR10, are endowed with a coiled-coil (CC) domain on their C-terminal ends. This domain is important for mediating interactions between members of the MTMR family (Kim et al.

2003; Berger et al. 2006; Choudhury et al. 2006). MTMRs have various other functional domains which can be used for potential protein-protein interactions (PDZ domain), protein-PIs interactions (FYVE, PX, PH domains) or Rab GTPase effectors (DENN domain) (Zhao et al. 2000; Itoh and Takenawa 2002; Lemmon 2003; Lee and Zheng 2010; Yoshimura et al. 2010). Based on the level of sequence homology and protein domain structure MTMRs have been divided into 6 classes containing exclusively the active or dead proteins (**Fig. 3**).

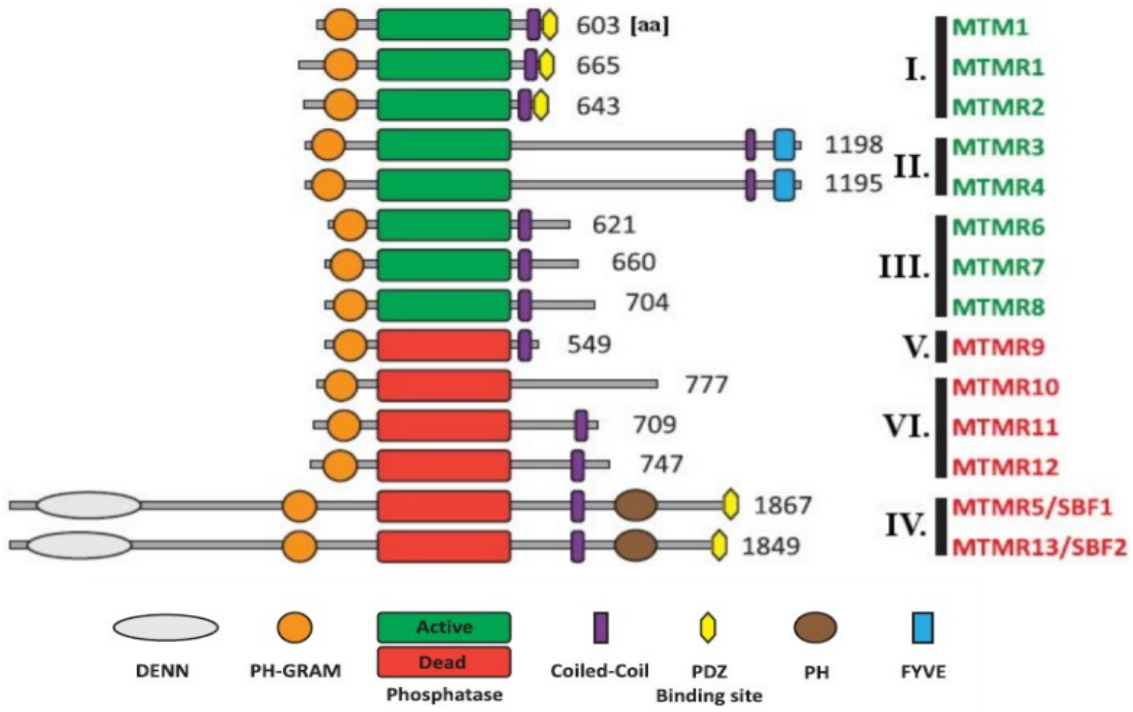


Figure 3: ***Organization of MTMRs protein domains.*** MTMRs are classified into two main groups of catalytically active and "dead" MTMRs. MTMRs are further divided into 6 subgroups (on the right) based on protein structural features (on the left). The picture was adjusted based on (Raess et al. 2017).

The central core

The crystallization of the central core of the active members MTMR2, MTMR8 and MTMR1 provided data for better understanding the MTMRs structure and function (Begley et al. 2003; Yoo et al. 2015; Bong et al. 2016). Crystal structure of MTMR6 was also done (www.rcsb.org, PDB ID 2FY0), however the data were not published yet. These structural data show that MTMRs PTP domain is significantly wider and deeper compared to other PTPs, thus enabling accommodation of the large headgroups of the PI substrates. This domain contains several hydrophobic residues that interact with the glycerol moiety of PIs and therefore allows partial penetration of the membrane upon substrate binding (Begley et al. 2003; Yoo et al. 2015; Bong et al. 2016). PTP domain appears to have an additional role in mediation of interactions

between the MTMR members (Yoo et al. 2015). PTP and CC domains were suggested to cooperate in mediation of multi-MTMRs complex interactions as shown in **Fig. 4**.

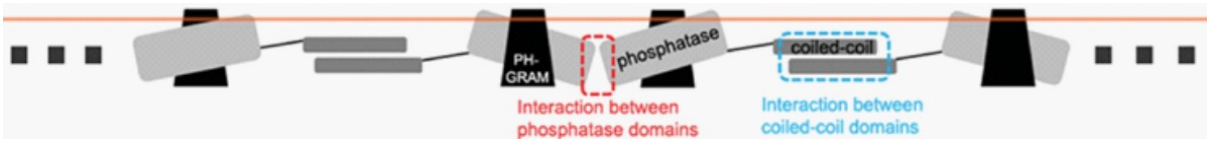


Figure 4: ***Proposed mechanism for MTMRs interactions on membranes.*** In this hypothetical model MTMRs employ CC domain and PTP domain to mediate multimeric interactions with other members. Through multimerization, MTMRs increase the affinity of binding to membrane PIs, mediated by PH-GRAM domain (The picture was adopted from (Yoo et al. 2015))

Initially discovered GRAM domain was shown to be part of a larger domain with the general fold characteristics for the PH domains, therefore it was renamed to PH-GRAM domain (Doerks et al. 2000; Begley et al. 2003). Similarly to PH domains, PH-GRAM domains mediate interactions with the membrane PIs (Doerks et al. 2000; Lemmon 2003; Berger et al. 2003; Choudhury et al. 2006; Tsuji et al. 2019). Sequence differences exist between the PH-GRAM domains of individual MTMR members suggesting their variable functions in the membranes (Choudhury et al. 2006; Bong et al. 2016). The generally low-affinity PH-GRAM membrane binding was suggested to be multiplied by the oligomerization interactions among the MTMR members (**Fig. 4**).

PTP and PH-GRAM domains are connected by a linker that forms an extensive interface between these two domains. The nature of the linker determines the general superposition of these two domains and sets the orientation of the PH-GRAM domain towards the membranes. PH-GRAM domain subsequently orients the neighbouring PTP domain favourably towards its substrates. Conformational deviations within the linkers therefore influence the specificity of MTMRs membrane binding (Begley et al. 2003; Yoo et al. 2015; Bong et al. 2016). It is worth mentioning that PTP/PH-GRAM proximal surface is electrostatically polarized with a positive charge. It helps to position the MTMRs towards the negatively charged headgroups of their PI substrates (Bong et al. 2016).

1.1.3. MTMRs function

The enzymatic activity of the MTMR members is linked to their transient localization to particular membranes (Robinson and Dixon 2006). When on a membrane, MTMRs regulate the levels of PtdIns3P and PtdIns(3,5)P_2 through their dephosphorylation to PtdIns and

PtdIns5P, respectively. The site of MTMRs action is therefore significantly determined by the occurrence of their substrates (Di and De 2006; Robinson and Dixon 2006).

PtdIns3P and PtdIns(3,5)P_2 are dominantly enriched in the membranes of the early and late endosomes, respectively, which suggests that MTMRs could play a role in endosomal trafficking (Di and De 2006; Lorenzo et al. 2006). Endosomal trafficking is coupled with many other cellular processes. It controls the nutrient uptake, secretion, signalling, adhesion, membrane homeostasis, immune response or apoptosis (Scott et al. 2014; Elkin et al. 2016). Accordingly, MTMRs have been implicated in many of these processes. MTMR3 and MTMR4 were shown to function in innate immune response control (Putri et al. 2019). MTMR6, MTMR9, and MTMR14 were linked to the control of apoptosis (Zou et al. 2009; Li et al. 2019). Besides the endosomes, the levels of PtdIns3P rise also in the membranes of the forming autophagosomes, which were also described as important sites of MTMRs function (Zou et al. 2012; Nascimbeni et al. 2017). Minor subpools of PtdIns3P might be present also in other cellular membranes. In concert with this, MTMR6 was observed to regulate Ca^{2+} activated K^+ channel in the PM in a PtdIns3P dependent manner (Srivastava et al. 2005). MTMR6 and MTMR9 were also described to regulate the ER-Golgi and intra-Golgi trafficking (Mochizuki et al. 2013; Doubravská et al. 2019). MTM1 was reported to function in the tubulation of membranes of both the sarcoplasmic reticulum and the PM in skeletal muscles (Amoasii et al. 2013; Royer et al. 2013). Previously, MTM1 was shown to localize to the Rac1 inducible ruffles at the PM (Laporte et al. 2002). In parallel, MTMR3 and its product PtdIns5P was seen involved in the regulation of cell migration also through regulation of Rac1 GTPase at the PM (Oppelt et al. 2013). Regarding the MTMRs role in cell migration, downregulation of MTMR3 and MTMR14 led to inhibition of migration in fibroblasts and cancer cell lines (Oppelt et al. 2013; Li et al. 2019). Unique roles for MTMR3 and MTMR4 were observed in the regulation of the late mitotic abscission through interactions with the key mitotic regulators PLK1 and CEP55, independently of the enzymatic activity (St-Denis et al. 2015).

MTMRs in endosomal trafficking

Extracellular cargos and surface molecules can be internalized into the cell through various mechanisms dominated by the clathrin-coated vesicle endocytosis. After the internalization, the cargos enter a dynamic network of tubulovesicular compartments called endosomes. This network ensures the cargo sorting and distribution to different final destinations. After the uptake, the vesicles start merging into the first compartment called the EE. At this place, the

initial protein sorting takes place. Proteins are either recycled back to the plasma membrane through the recycling endosomes, sent to the cis-GA through the retrograde transport, routed for degradation in the lysosome through maturation in multivesicular bodies and LEs or in polarized cells transported across the cells in a process called transcytosis (Elkin et al. 2016). Important aspect of endosomal protein sorting and trafficking is also determined by the membrane identity formed by the unique distribution of PI species and of proteins of the RAB family of small GTPases (Jean and Kiger 2012; Elkin et al. 2016). PIs and RAB GTPases co-work to mediate interaction between the cargos and the effector proteins. The effector proteins can promote membrane remodelling, endosomal tethering, docking and fusion, thereby enabling receptor sorting and endosomal transport. During the endosomal maturation towards lysosomal degradation, the early endosomal RAB5 and PtdIns3P are converted to the late endosomal RAB7 and PtdIns(3,5)P_2 (Wallroth and Haucke 2018). PIs and RAB GTPases are regulated with the dynamics that keep the membrane identity transitory. It is not surprising that the functions and regulations of PIs and RAB GTPases are often coupled. MTMRs were found to be both regulating and regulated by RAB GTPases (Jean and Kiger 2012; Jean et al. 2015; Mochizuki et al. 2013).

Several MTMRs were seen to localize to the same places and to be involved in similar pathways as their substrates. For example, MTMR4 was shown to co-localize with the markers of the EE (Lorenzo et al. 2006). MTMR4 activity on EE was coupled with the presence of PtdIns3P and RAB5 and RAB11 and MTMR4 overexpression impaired TNF receptor sorting for recycling back to the PM (Naughtin et al. 2010). Later study reported that MTMR4 localizes mainly to the LE and downregulates maturation of endosomes along the pathway to the lysosomes through control of PtdIns3P level (Pham et al. 2018). Its activity towards PtdIns3,5P2 was shown to be negligible (Teo et al. 2016; Pham et al. 2018). Similarly, MTM1 and MTMR2 were localized on the membranes of both the EE and LE (Tsujita et al. 2004; Franklin et al. 2013). Regarding MTMR2, its localization on the EE and its subtypes was shown to be dependent on the phosphorylation state of this protein. MTMR2 phosphorylation state was shown to be regulated through the MAPK signalling (Franklin et al. 2013). Another research revealed that induced activity of MTMR2 on the membrane of the EE temporally downregulated the occurrence of Receptor mediated endocytosis 8 (RME-8) protein in the membrane of the EE. This protein is involved in clatrin-mediated endocytosis (Xhabija et al. 2011). The levels of PtdIns(3,5)P_2 raise upon stimulation by conditions such as hypoosmotic shock or EGFR stimulation (Tsujita et al. 2004; Jin et al. 2016). Similarly, stimulation of EGFR

and hypoosmotic shock resulted in the re-localization of MTM1 and MTMR2 to the membranes of the LEs, the sites characteristic by higher levels of PtdIns(3,5)P_2. Their overexpression impaired EGFR degradation in the lysosome and led to the formation of enlarged vacuoles (Berger et al. 2003; Tsujita et al. 2004; Cao et al. 2008; Berger et al. 2011a; De Craene et al. 2017). Although multiple MTMRs have regulatory function alongside the endocytic pathway, they roles appear to be non-redundant (Laporte et al. 2002; Cao et al. 2008; Zou et al. 2012; Mammel et al. 2019). The MTMRs specificity is achieved by a combination of several regulatory mechanisms described below.

MTMRs in autophagy

The macroautophagy, simply termed as autophagy, is an evolutionary conserved self-degradation pathway. During autophagy, cellular components are embraced by a double membrane vesicle called an autophagosome which ships the load to the lysosome for degradation and recycling. Basal autophagy runs in the cell under standard conditions to protect the cell from damaged proteins and organelles. The process is increased in the state of nutrient stress to balance the lack of the energy sources (Glick et al. 2010; Hansen and Johansen 2011). During starvation, autophagy is induced due to downregulation of mTOR (target of rapamycin) complex 1 (mTORC1), the key inhibitor of autophagy (Shang et al. 2011). Autophagy is executed by a sequential action of several ATG proteins. The initiation of autophagy is related to the formation of a structure called an omegasome at the membrane of the ER rich for PtdIns3P. The omegasome transforms into pre-autophagosomal isolation double membrane also known as the phagophore. PtdIns3P serves as a platform for recruitment of ATG12-5-16L conjugation complex which leads to elongation of the phagophore membrane. This complex also enables formation of subsequent LC3-PE complex, which was suggested to function in the closure of the phagophore and genesis of the complete autophagosome. The final step of autophagosome metabolism is its fusion with lysosome followed by degradation (Shibutani et al. 2015; Dudley et al. 2020).

Consistent with the role of PtdIns3P in autophagy, manipulation of several MTMRs, the key regulators of PtdIns3P, appeared to dysregulate the autophagic markers. MTM1, MTMR3, MTMR4, MTMR6, MTMR14 and complex of MTMR8/MTMR9 were shown to downregulate the initial step of autophagosome formation through depleting PtdIns3P from the ER membrane (Vergne and Deretic 2010; Taguchi-Atarashi et al. 2010; Zou et al. 2012; Mochizuki et al. 2013; Al-Qusairi et al. 2013). Besides its enzymatic activity, MTMR3 showed an additional

regulatory role upstream of the autophagic pathway, particularly through the inhibition of the protein complex mTORC1, the master regulator of autophagy (Hao et al. 2016). Some MTMRs, specifically MTM1, MTMR4; MTMR13 and MTMR14, were suggested to function also in a later step of autophagy, namely during the autophagosomal-lysosomal fusion (Al-Qusairi et al. 2013; Pham et al. 2018; Jean et al. 2015; Gibbs, Feldman, a Dowling 2010). Interestingly, MTMR13 regulates the late step of autophagy at the level of endosomal trafficking. MTMR13 controls the endosomal trafficking of the fusion snare protein VAMP8. VAMP8 is required for the autophagosomal-lysosomal fusion, dependently on its GEF activity executed on the EE RAB21 GTPase (Jean et al. 2015). For the remaining MTMRs the precise mechanism of their function in the autophagosome maturation remains to be explored. Based on these observations a multilevel regulation of the autophagy pathway by MTMRs is highly expectable.

MTMRs in diseases

Although all members of the MTMR family share their substrate specificity, they roles are not redundant in both the cell and human organism as such (Raess et al. 2017). The first member of the MTMR family was found in 1996 mutated in X-linked myotubular myopathy. This disease is affecting the muscle tissue and is characterized by severe hypotonia and muscle weakness, derived from impaired maturation of muscle fibres (Laporte et al. 1996). Few years later, another member MTMR2 was found to be mutated in autosomal recessive neuropathy called Charcot-Marie-Tooth syndrome type 4B1 (CMT4B) (Bolino et al. 2000). Unlike the X-linked MTM, CMT-4B is affecting peripheral nervous system (PNS) and is characterized by demyelination of the nerves with focally placed myelin sheets. This malfunction leads to a progressive sensory loss (Bolino et al. 2000). In the following years, different MTMRs were linked to other pathologies such as various types of cancer, higher risk of the metabolic syndrome, susceptibility to the Creutzfeld-Jakob disease or male infertility (Firestein et al. 2002; Hotta et al. 2011; Sanchez-Juan et al. 2012; Tang et al. 2014; Zheng et al. 2014; Weidner et al. 2016; Yuan et al. 2017; Wen et al. 2018; Wang et al. 2019). The fact that malfunctions of individual members of MTMR family result into many different pathologies supports the idea of the specific functions of the MTMR members. Interestingly, some MTMR members were linked to the subtypes of the same diseases as seen for example for MTMR2, MTMR13/Sbf2 and MTMR5/Sbf1. Mutations in these members cause CMT type 4B1, 4B2 and 4B3, respectively. These observations suggest that there might be a relationship between

these members. Indeed, inactive members MTMR5 and MTMR13 were shown to form a hetero-complex with the active MTMR2 in the PNS (Mammel et al. 2019).

1.1.4. MTMRs specificity

The roles of the individual MTMR proteins are not redundant. The specificity of MTMRs function is closely related to the way they are being regulated. Different regulatory mechanisms are employed to control MTMRs localization and function within the cell and tissue (Raess et al. 2017).

Tissue expression

Even though most of MTMRs are ubiquitously expressed, the intensity of their expression fluctuates across the tissues but also in time (Raess et al. 2017). For example, MTMR5 and MTMR13 are both being expressed in the PNS where they interact with the same active partner MTMR2. However, their expression levels peak in different stages of embryogenesis. Their time-dependent expression determines their specific functions (Mammel et al. 2019).

Alternative splicing

MTMRs specificity might be also supported by alternative splicing. It was shown that the effect of specific tissue expression and alternative splicing can be coupled. As an example, Buj-Bello and colleagues observed an altered mRNA splicing for MTMR1 in patients with congenital myotonic dystrophy (cDM1) (Buj-Bello et al. 2002). Their study suggests a muscle specific expression for a *MTMR1* isoform in a time-dependent manner during muscle formation. Simple searching in the ENSEMBL database (www.ensemble.org) revealed that each MTMR gene has multiple protein coding and non-coding isoforms. Although some studies on MTMRs took the existence of their isoforms into account (Buj-Bello et al. 2002; Mochizuki et al. 2013), majority of the literature up to nowadays has been working with the most canonical gene variants (Zou et al. 2009; Zou et al. 2012; Robinson et al. 2018; Doubravská et al. 2019). Alternative splicing represents yet poorly explored regulatory mechanism for MTMRs function.

Specific interactions

Individual members of MTMR family have their own spectrum of interacting molecules made of proteins and lipids which strongly determines their function (St-Denis et al. 2015). The interaction network for each member can vary with respect to the tissue of expression, where

different binding partners are available. As an example, MTMR2 was shown to interact with different proteins in Schwann cells and in the peripheral sensory neurons. Through these specific interactions, MTMR2 regulates different processes in these cells (Bolis et al. 2009; Narayanan et al. 2018).

Besides the tissue expression, the specificity of MTMRs interactions is strongly dependent on their protein structure. As shown in **Fig. 3**, the members of MTMR family may contain extra domains, usually shared within a particular subgroup. For example, members of subgroup IV, MTMR5 and MTMR13, contain the GEF homology DENN domain. Consistently, MTMR13 was shown to regulate RAB21 GTPase through this domain (Jean et al. 2015). MTMRs structural heterogeneity can be also found within the more common, generally shared domains. For instance, MTMR6 was shown to specifically interact with KCa3.1 ion channel on the PM through its CC and PH-GRAM domains and to regulate its function. Chimeric MTMR2 and MTMR8 proteins including MTMR6 CC and PH-GRAM domains resulted in the same function (Srivastava et al. 2005). Also, important structural differences can be found within the flexible parts of MTMRs. The character of the linker between the PTP and PH-GRAM domain determines the superposition of these domains together, which influences the binding surface of the protein (Yoo et al. 2015; Bong et al. 2016).

The structure or function of MTMRs can be additionally modified by posttranslational modifications. Among the types of modifications, phosphorylation is the most prevalent. Phosphorylation can either induce conformational changes within the protein or modulate its binding properties and thereby its localization or function (Cheng et al. 2011). Illustratively, MTMR2 phosphorylation at Ser 58 by MAPK (Erk1/2) sequesters MTMR2 from membrane of the EE. Further phosphorylation at Ser631 targets MTMR2 to the APPL-1 rich EE subtype. Therefore localization of MTMR2 to the specific membranes, containing subpools of PtdIns3P, is modulated by its phosphorylation state (Franklin et al. 2013). It is expectable that other members could be regulated by this modification in a similar manner. Besides phosphorylation, ubiquitination is also among the most prevalent protein modifications, usually controlling protein degradation. However different ubiquitination patterns were observed to regulate more cellular processes, including endocytosis and trafficking of membrane proteins or multiple signalling pathways (Foot et al. 2016). Although ubiquitination has not been described yet in the context of MTMRs regulation, its putative role cannot be excluded. Membrane binding proteins can also be specifically lipidated, e.g. by palmitoylation or other modifications facilitating their association with the membrane (Ray et al. 2017). MTMRs are cytosolic

proteins, which transiently bind to various membranes to execute their function (Robinson and Dixon 2006). It is therefore tempting to hypothesize that MTMRs membrane binding could be supported by reversible lipidation.

Overall, the specificity of MTMRs is supported by their unique interactions. The specific interactomes are determined by combinations of the tissue expression, the specific structure of the protein and putative posttranslational modifications. Interestingly, interactomes of various MTMRs often include specific lipid kinases and small GTPases of the RAB family. In case of MTM1, the protein interacts with hVPS5/hVPS34 lipid kinase complex on the early endosomes to execute its function (Cao et al. 2007). MTMR13 coordinates the function of a small GTPase RAB21 in the endocytic and autophagic pathways (Jean and Kiger 2012; Jean et al. 2015). MTMR6 is being regulated by small RAB1 GTPase in the early secretory pathway (Mochizuki et al. 2013). Some MTMRs were also shown to be allosterically regulated by their product PtdIns5P (Schaletzky et al. 2003; Lorenzo et al. 2005; Zou et al. 2009). Additionally, MTMRs form multiple interactions with other members of MTMR family. Strong regulatory potential lays in these interactions (Raess et al. 2017). Through these hetero-interactions, the inactive MTMRs were shown to influence the localization, stability and function of their active partners (Robinson and Dixon 2006; Zou et al. 2012; Raess et al. 2017; Doubravská et al. 2019; Mammel et al. 2019)

1.2. The role of inactive MTMRs

The main role of the catalytically inactive MTMRs is based on their ability to interact with the active partners and to regulate their localization and function in the cell. Although the precise mechanisms through which the inactive MTMRs execute their regulatory roles are not yet fully understood, several studies were made to help uncover this topic (Robinson and Dixon 2006; Zou et al. 2009; Zou et al. 2012; Doubravská et al. 2019; Mammel et al. 2019). Moreover, some inactive MTMRs have additional intrinsic roles, for example acting as GEFs (Jean et al. 2015).

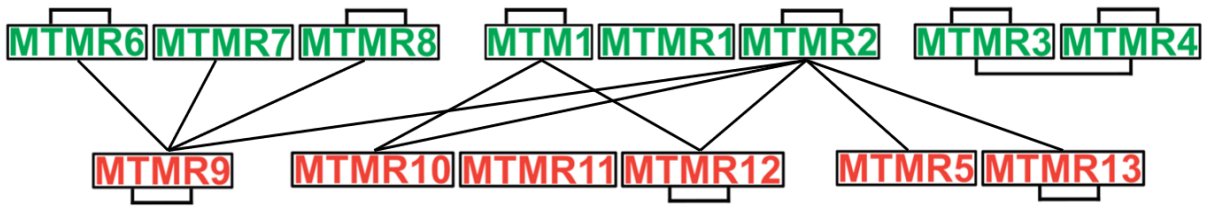


Figure 5: Described interactions between members of the MTMR family. The active MTMRS (green) and inactive MTMRs (red) form multiple homo- and hetero- interactions within the MTMR family (Inspired by Raess 2017; St.-Denis 2015)

Among the 14 regular members of the MTMR family, 20 interactions were published up today (**Fig. 5**). Two main domains are involved in these interactions - the CC domain and the PTP domain (Berger et al. 2003; Kim et al. 2003; Schaletzky et al. 2003; Srivastava et al. 2005; Choudhury et al. 2006; Lorenzo et al. 2006; Yoo et al. 2015). The level of structural similarity in the CC domains corresponds with the higher chance of binding (Srivastava et al. 2005; Choudhury et al. 2006). Several members were shown to form homodimer interactions. Additionally, MTMR members form multiple hetero-interactions mediated mostly between the active and inactive members (Raess et al. 2017). Notably, the members of the same subgroup often share the interaction partners. For that reason, the role of inactive MTMRs is described separately for each of the three inactive subgroups.

1.2.1. Subgroup IV

MTMR subgroup IV contains two catalytically inactive members MTMR5/Sbf1 and MTMR13/Sbf2, hereafter simply termed as MTMR5 and MTMR13. These proteins share a high level of protein organization including the extra domains – C-terminal PH domain and N-terminal GEF homology DENN domain (**Fig. 3**). They also share the interaction partner, the enzymatically active member MTMR2, extensively studied for its relation with the CMT neuropathy type 4B1 (Kim et al. 2002; Bolino et al. 2004; Robinson and Dixon 2005). In the course of time, it was found out that the mutations in MTMR13 and MTMR5 cause different types of the same pathology – the CMT type 4B2 and 4B3, respectively (Senderek et al. 2003; Nakhro et al. 2013). These clinical manifestations indicated that the functions of the inactive MTMR5 and MTMR13 and the active MTMR2 might be coupled (Bolino et al. 2000; Senderek et al. 2003; Nakhro et al. 2013). Indeed, MTMR5 and MTMR13 were shown to form hetero-complexes with MTMR2 during the development of PNS to control proper axonal radial sorting and Schwan cell myelination (Mammel et al. 2019). The non-redundant function of these two complexes appeared to be determined by different time expressions during embryogenesis (Mammel et al. 2019). The mode of interaction was described on the MTMR2/MTMR13 complex (Berger et al. 2006). The interacting proteins first form homodimers dependently on the presence of the CC domains. Next, the pre-formed homodimers then employ another domain to create a hetero-tetramer complex as shown in **Fig.6**.

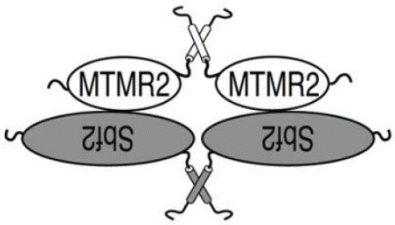


Figure 6: Model of MTMR2/MTMR13 hetero-tetramer interaction. MTMR2 and MTMR13 homodimerize through CC domains and further interact to form the hetero-complex. (Adopted from (Berger 2006)).

The interacting proteins in the MTMR2/MTMR5 and MTMR2/MTMR13 complexes were described to reciprocally stabilize each other, except for MTMR5, which appeared to have no influence on the MTMR2 protein levels. These stabilizations were shown to be exclusive for the PNS (Ng et al. 2013; Mammel et al. 2019). The phenotype seen in patients with CMT-4B1 was suggested to be due to the elevated levels of PtdIns(3,5)P_2 as observed in the mice model deficient for MTMR2 (Vaccari et al. 2011). In harmony with this, overexpression MTMR2 led to inhibition of EGFR degradation and formation of enlarged vacuoles. MTMR13 was observed to oppose this effect (Berger et al. 2011b). Both MTMR5 and MTMR13 were previously observed *in vitro* to increase the enzymatic activity of MTMR2 (Kim et al. 2003; Berger et al. 2006). However, manipulation with protein kinases producing PtdIns3P, the direct precursor of PtdIns(3,5)P_2 did not influence the prevalence of the myelin outfoldings (Robinson et al. 2018). It was suggested that MTMR13 has an additional function required for the proper formation of myelin in Schwann cells, potentially acting as a scaffold molecule or GEF and that this function might be potentially regulated by the active MTMR2 (Robinson et al. 2018). The GEF activity of MTMR13 was previously observed in HeLa cells upon starvation-induced autophagy. The GEF activity of MTMR13 was previously observed in HeLa cells upon starvation-induced autophagy. Through activating small GTPase RAB21 at the membranes of the EE, MTMR13 regulated trafficking of the SNARE protein VAMP8 to the membranes of the late endosomes and lysosomes, where VAMP8 is required for the autophagosomal-lysosomal fusion (Jean et al. 2015).

1.2.2. Subgroup V

MTMR subgroup V contains only a single member, the inactive phosphatase MTMR9. MTMR9 forms hetero-interactions with all the three members of MTMR subgroup III, namely

MTMR6, MTMR7 and MTMR8. While subgroup III members utilize MTMR9 as their exclusive inactive partner, MTMR9 is further capable to bind with the catalytic active MTMR2 or to form a MTMR9 homodimer (St-Denis et al. 2015; Raess et al. 2017). The binding with MTMR9 can be competitive among members of subgroup III. Simultaneously the binding result in stabilization of both proteins in the complex (Zou et al. 2012).

Overexpressed MTMR9 was observed to localize into the intermediate compartments (IC) and the GA (Doubravská et al. 2019). Moreover, it recruits co-expressed MTMR6 and MTMR8 to the same compartments (Doubravská et al. 2019). Although MTMR9 was shown to accelerate the phosphatase activity of MTMR6 and MTMR8 towards both their substrates, MTMR8/MTMR9 complex significantly preferred PtdIns3P and inhibited starvation-induced autophagy (Zou et al. 2009; Zou et al. 2012). Oppositely, MTMR6/MTMR9 complex preferred PtdIns3,5P2 and served as an inhibitor of stress-induced or etoposide-induced apoptosis. MTMR9 thus regulates MTMR6 and MTMR8 substrate specificity (Zou et al. 2012). The effect of MTMR9 on autophagy was also reported, when the knock-out (KO) of MTMR9 promoted an increase in the basal autophagy (Doubravská et al. 2019). When overexpressed, MTMR9 markedly co-localized with RAB1 GTPase on the IC and the GA and regulated its distribution (Doubravská et al. 2019). Similar co-localization and function was observed for MTMR8 (Doubravská et al. 2019). MTMR6 was previously described to control an early step of autophagy through interaction with RAB1 on membranes of the IC and the GA in rat kidney cells (Mochizuki et al. 2013). However, later study had not confirmed MTMR6 co-localization with RAB1 in RPE-1 cells (Doubravská et al. 2019). Explanation for this could reside in the fact, that MTMR8 is not expressed in rodents, where its absence could be compensated by MTMR6 taking over some of its roles (Mochizuki et al. 2013; Doubravská et al. 2019). The experiment with KO of MTMR9 resulted in redistribution of RAB1 and the actin nucleation promoting factor WHAMM and in disintegration of the GA. The sequence and continuity of these events was, however, not explored. MTMR9 role was simultaneously suggested to positively regulate the secretory pathway, since a representative secretory protein WNT3A showed decelerated secretion in the cells with MTMR9 KO (Doubravská et al. 2019).

From the MTMR9 interaction partners, the MTMR7 is the most unique among the MTMR family. In contrast to other MTMRs, MTMR7 is express almost exclusively in the brain neuronal cells, where it localizes to the cytosol and to Golgi-like granules (Mochizuki and Majerus 2003). Although MTMR7 is capable to dephosphorylate PtdIns3P, its preferred substrate is soluble inositol 1,3-bisphosphate ($Ins(1,3)P_2$) (Mochizuki and Majerus 2003).

MTMR9 was shown to form complex with MTMR7 via the CC domain. Through this interaction, MTMR9 increased MTMR7 phosphatase activity towards Ins(1,3)P_2. MTMR9 and MTMR7 were described to reciprocally stabilize each other in the neuronal cells (Mochizuki and Majerus 2003).

1.2.3. Subgroup VI

MTMR subgroup VI contains three inactive MTMRs, namely MTMR10, MTMR11, and MTMR12. MTMR11 is the only inactive member of the MTMR family, which has not been seen to form any hetero-interactions with other MTMR members (Raess et al. 2017). In contrast, MTMR10 and MTMR12 share the interaction partners MTM1 and MTMR2, the two active phosphatases from subgroup I (Nandurkar et al. 2003; Lorenzo et al. 2006; Raess et al. 2017). MTM1 and MTMR2 are clinically important proteins, since their malfunctions result in serious hereditary diseases, thereby MTMR10 and MTMR12 are of interest as their potential regulators (Laporte et al. 1996; Bolino et al. 2000).

MTMR12 was first found as an adaptor subunit named 3-PAP that co-precipitated with MTM1 (Nandurkar et al. 2001). Further study revealed that overexpressed MTMR12 binds with MTM1 and MTMR2 (Nandurkar et al. 2003). Through the interaction MTMR12 regulated MTM1 re-localization from the PM into the cell cytoplasm in COS-7 cells (Nandurkar et al. 2003). Further studies in zebrafish and mammalian cells revealed that these proteins reciprocally increase protein stability of themselves the skeletal muscles (Gupta et al. 2013). Consistently, XLMTM patients with mutations disrupting MTMR12-MTM1 binding had the level of both these proteins significantly reduced. Simultaneously, experiments with KO MTMR12 in zebrafish resulted in similar skeletal muscle defects as observed in patients with XLMTM (Gupta et al. 2013). These observations indicate, that MTMR12 forms a stable complex with MTM1 in skeletal muscles (Gupta et al. 2013). It is tempting to hypothesize that through this interaction MTMR12 regulates proper function of MTM1 in skeletal muscles.

So far, the function of MTMR10 member has not been explored yet.

2. Objectives

Active MTMRs were shown to be regulated through interactions with their inactive partners. Defects in MTM1 and MTMR2 cause XLMTM and CMT, serious hereditary disease with estimated incidence 1:50 000 males and 1:2500 new-borns, respectively, as stated in the Genetics Home Reference (www.ghr.nlm.nih.gov). So far MTMR2 was seen to be regulated by the inactive phosphatases MTMR5 and MTMR13 during development of PNS (Mammel et al. 2019). MTM1 was described to form stable complex with MTMR12 in skeletal muscles and the regulatory role of MTMR12 strongly suggested (Gupta et al. 2013). Whether and how MTMR10 and MTMR12 act to regulate their active partners MTM1 and MTMR2 remains to be elucidated. We set the following goals to answer these questions:

- To define the sites of MTMR10 and MTMR12 intracellular localization.
- To investigate whether particular posttranslational modifications of MTMR10 and MTMR12 influence the subcellular localization of these proteins.
- To analyse the behaviour of MTMR10 and MTMR12 upon induction of processes accompanied by elevated levels of PtdIns3P, the substrate of MTMRs.
- To explore, whether co-expression of MTM1 and MTMR2 with MTMR10 and MTMR12 results in the re-localization of participating MTMRs.

3. Results

MTMR10 and MTMR12 are two catalytically inactive phosphatases from the family of MTMRs. These proteins interact with MTM1 and MTMR2, the two active members of MTMR family associated with hereditary diseases XLMTM and CMT-4B, respectively (Raess et al. 2017). Understanding of MTM1 and MTMR2 way of regulation is of high interest and could potentially advance the XLMT and CMT-4B therapeutic treatment. Whether and how MTMR10 and MTMR12 function to regulate MTM1 and MTMR2 is yet poorly understood. We decided to explore this topic by the experiments described below.

Since several isoforms were described for most MTMRs (Raess et al. 2017), we initially aimed at cloning the three known protein-coding isoforms of MTMR10 and MTMR12 to work with using ENSEMBLE database (www.ensembl.org; ENSG00000166912 ;ENSG00000150712). However, for both MTMR10 and MTMR12 only the longest, canonical isoform was obtained by PCR from cDNA isolated from SHS and RPE-1 cell lines, respectively. The MTMR cDNAs were cloned into the final vectors MTMR10/pcDNA4.0TOMycHis and MTMR12/pEGFP-N3 using restriction cloning approach. It was empirically discovered in our group that the proteins from the MTMR family are functional when expressed with a tag on their C-terminal end (data not shown). Therefore, *MTMR10* and *MTMR12* protein coding sequences were inserted in the vectors in the directions ensuring the attachment of the MycHis and EGFP tags to their C-terminal ends.

3.1. MTMR10 localizes to the cytoplasm

To assess the role of MTMR10 protein in the cell, we intended to examine the localization of overexpressed MTMR10-MycHis in mammalian cells and to explore the potential influence of the selected posttranslational modifications on their localization pattern. Firstly, we wanted to verify that MTMR10-MyHis protein is being produced. For that, we transfected HEK293 cells with MTMR10/pcDNA4.0TOMycHis and confirmed the MTMR10-MycHis protein expression (**Fig. 7A**). To describe where the MTMR10 protein localizes in the cell, we transfected HeLa cells with the same vector and processed the cells for the confocal microscopy. As shown in **Fig. 7B**, MTMR10-MycHis was distributed through the cytoplasm with occasional enrichment at the PM.

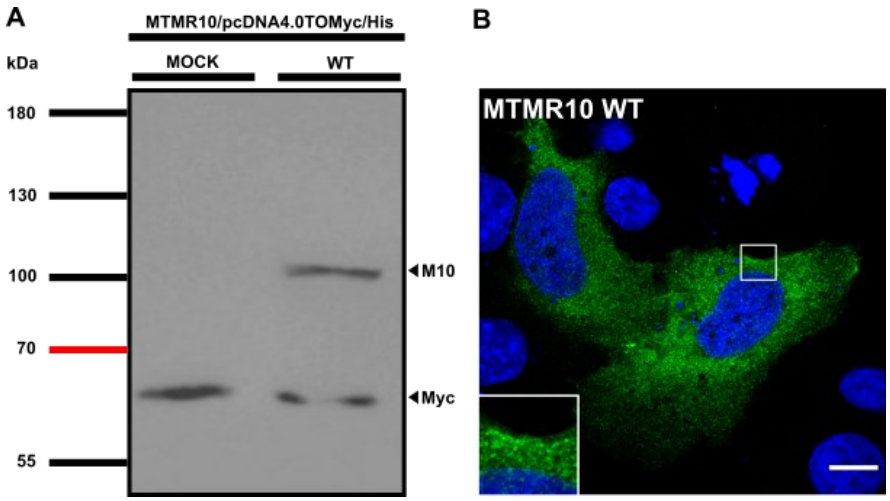


Figure 7: ***Overexpressed MTMR10-MycHis is distributed through the cell cytoplasm.*** **A)** MTMR10-MycHis (89 kDa) is being produced in HEK293 cells. Endogenous Myc staining (49 kDa) serves as a loading control. **B)** Overexpressed MTMR10-MycHis is dispersed through the cytoplasm of HeLa cells with occasional localization at the PM; Scale bar: 10 μm. Single scan picture was acquired using confocal microscope Leica TSC SP2.

As reported previously, MTMR protein localization and function can be regulated by posttranslational modifications (Franklin et al. 2013). To explore whether MTMR10 localization is modulated this way, we selected 6 potentially interesting modifications based on combined data from predictions from the software tool CSS-Palm (csspalm.biocuckoo.org) and the mass spectrometry data from the PhosphoSitePlus database (phosphosite.org). Cystein (Cys/C)411 was used as a possible candidate for palmitoylation, serin (Ser/S)607, tyrosine (Tyr/Y)708 and Ser751 as candidates for phosphorylation and lysin (Lys/K)611 and Lys613 as candidates for ubiquitination (**Fig. 8**). To examine the role of these modifications, we disrupted potential effect of these modifications by replacing the corresponding amino acids using directed PCR mutagenesis. With respect to stereochemical properties of substituted amino acids, Cys was replaced by Ser, Ser and Tyr by Ala and Lys by Arg. Mutated vectors were verified by sequencing. Unfortunately, when we tried to verify the mutant MTMR10 protein expression, we failed to detect the expression of both the mutated MTMR10 proteins and the original WT MTMR10 in HEK293 cells. Although the sequencing had not revealed any unintended mutations, all the subsequent Western blot experiments were repeated with the same results. Therefore, the experiments with MTMR10 were postponed and the attention was directed to MTMR12.

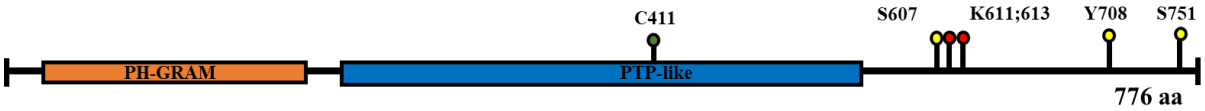


Figure 8: ***Selected posttranslational modifications shown in the context of MTMR10 structure.***
Palmitoylation – green; Phosphorylation – yellow; Ubiquitination – red. (Inspired by (Raess. et al. 2017)).

3.2. MTMR12 localizes to the cytoplasm and to the plasmatic membrane independently of selected posttranslational modifications

Our next interest was to characterize the MTMR12 protein. We aimed to study the localization of MTMR12 in the cell and to describe the putative effect of selected posttranslational modifications on the distribution of the protein. For the experiments, MTMR12 cDNA was inserted into two different vectors, pcDNA4.0TOMycHis and pEGFP-N3. Using restriction cleavage approach at first, the protein coding sequence was successfully inserted only into the pEGFP-N3 vector. MTMR12/pcDNA4.0TOMycHis was subcloned later using Gibson assembly approach (**Fig. 13A**), therefore the following experiments were done using the MTMR12/pEGFP-N3 vector only.

In order to verify the MTMR12-EGFP protein expression, we transfected the HEK293 cells with MTMR12/pEGFP-N3 and using Western blot approach, we confirmed that MTMR12-EGFP protein is being produced in HEK293 cells (**Fig. 9A**). Next, we investigated the MTMR12 localization within the cell. For that we overexpressed MTMR12-EGFP in the immortalized non-carcinoma cell line RPE-1 and observed that the protein is mainly present in the cell cytoplasm with local enrichment at the PM (**Fig. 9B**). The experiment was repeated in HeLa cells with the same results (data not shown), nevertheless, for all the subsequent experiments we decided to use RPE-1 cell line as the preferred option.

To inspect whether MTMR12 localization can be affected by some posttranslational modifications, we selected four potentially interesting modifications using predictions and mass spectrometry data as described above for MTMR10. Lys136 was chosen as possible candidate for ubiquitination, Cys398 as a possible candidate for palmitoylation and S564 and S716 as possible candidates for phosphorylation (**Fig. 10**). Interestingly, when detecting MTMR12-EGFP or MTMR12-MycHis by Western blot approach, in some cases we observed two MTMR12 bands on the blotting membrane (**Supplementary fig. 1A; Fig. 13A**). This

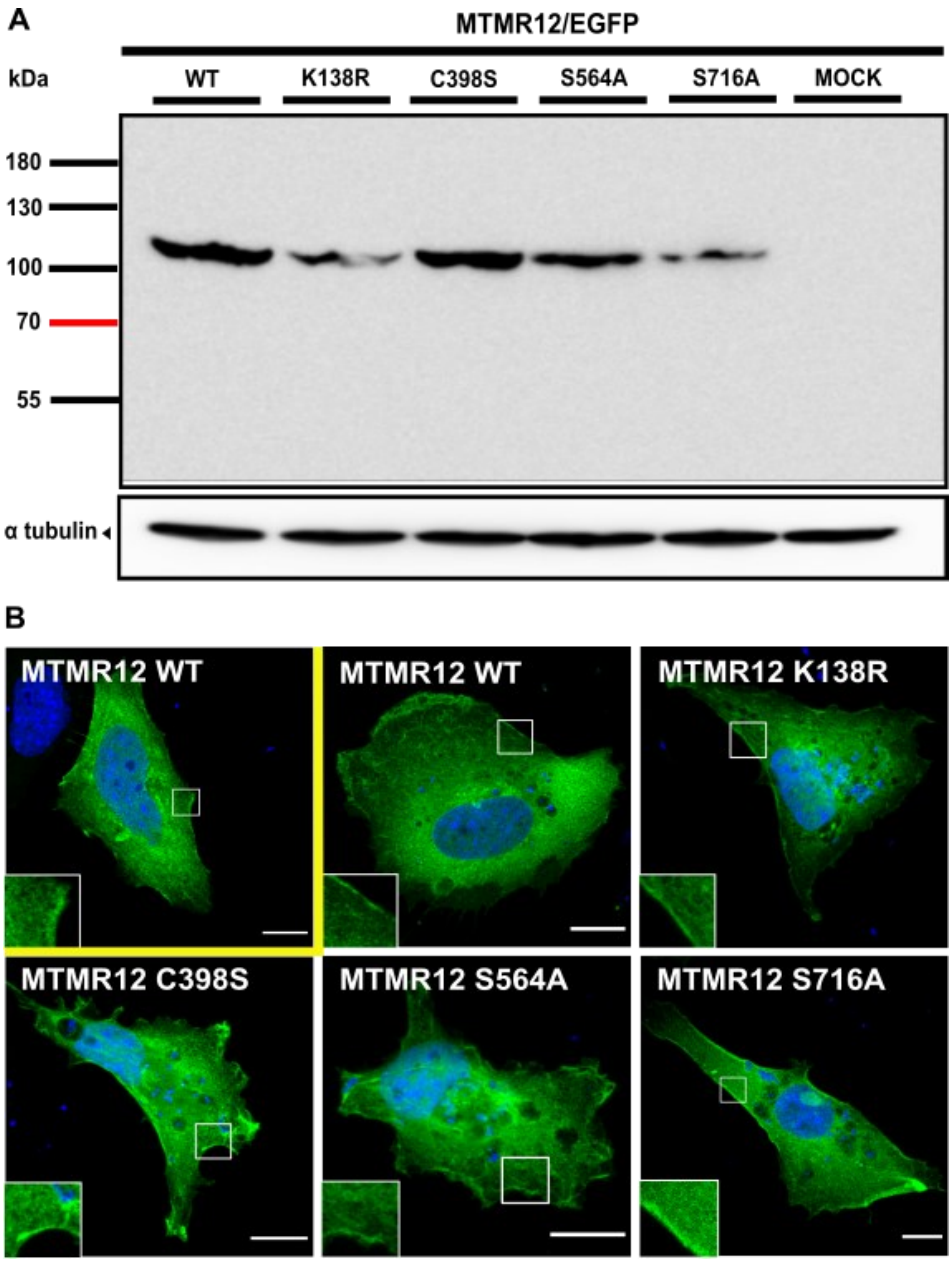


Figure 9: ***Overexpressed MTMR12-EGFP is distributed between the cytoplasm and the plasmatic membrane independently of selected posttranslational modifications*****. A)** MTMR12-EGFP (113 kDa) is being produced in HEK293. α-tubulin (50 kDa) serves as a control. **B)** Overexpressed WT MTMR12-EGFP localizes to the cell cytoplasm and at the plasmatic membrane in HeLa (yellow square) and RPE-1 (other square) cells. Mutations of selected posttranslational modifications do not influence localization of MTMR12-EGFP in RPE-1 cells (remaining squares.); Scale bar: 10 μm.

could indicate that indeed some posttranslational modifications exist on MTMR12. To analyse the effect of proposed modifications, we disrupted their potential influence by site-directed PCR mutagenesis of the corresponding amino acids. The experiment was designed in the same manner as for MTMR10, we confirmed that the modified proteins are being produced in the HEK293 cells using Western blot approach (**Fig. 9A**). We then overexpressed the mutants in RPE-1 cells and observed their localization. The localization pattern of mutated proteins was

highly similar to that of the WT, mutated MTMR12-EGFP was mostly present in the cell cytoplasm and at the plasmatic membrane (**Fig. 9B**). This indicates that the selected posttranslational modifications themselves do not have any effect on the cytoplasmic/PM localization of the MTMR12 protein in the cell.

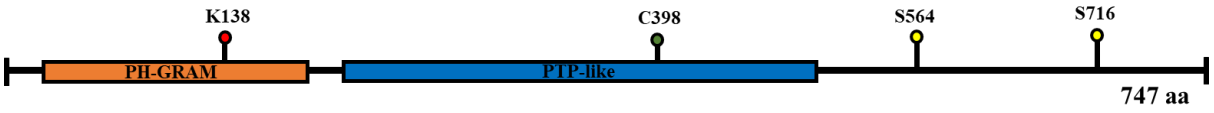


Figure 10*: Selected posttranslational modifications shown in the context of MTMR12 structure:* Palmitoylation - green, Phosphorylation - yellow, Ubiquitination – red. (Inspired by (Raess 2017)).

3.3. MTMR12 associates with the early autophagic markers upon induction of starvation independently of selected posttranslational modifications

Our next goal was to explore whether induction of increased levels of PtdIns3P, the substrate of MTMRs, results in altered localization of MTMR12. We also asked whether the selected posttranslational modifications affect MTMR12 behaviour upon such conditions. Firstly, we transfected RPE-1 cells with WT MTMR12-EGFP and exposed them to 4 h of starvation in order to induce cell autophagy. Upon these conditions, WT MTMR12-EGFP showed clear re-localization into undefined starvation-induced structures (**Fig. 11**). To explore whether selected protein modifications play role in this process, all the mutated vectors were transfected into RPE-1 cells and the transfected cells were then starved for 4 h (**Fig. 11**). The localization of the mutated proteins was comparable with WT MTMR12-EGFP phenotype, suggesting that the selected protein modifications do not play an important role in the starvation induced re-localization of MTMR12 protein. To sum up, MTMR12 re-localizes into undefined vesicle-like structures upon starvation of RPE-1 cells independently of the selected posttranslational modifications.

To characterize the MTMR12-positive structures observed upon cell starvation, several markers were chosen for further co-localization experiments. Our primary interest was whether the re-localization of MTMR12-EGFP upon starvation is connected to the process of autophagy. To find out we stained the cells for markers of autophagosome initiation (ATG12, ATG16 protein markers) and formation (LC3B protein marker.) Co-localization experiments showed association between MTMR12-EGFP and ATG12-positive structures upon starvation (**Fig. 12**).

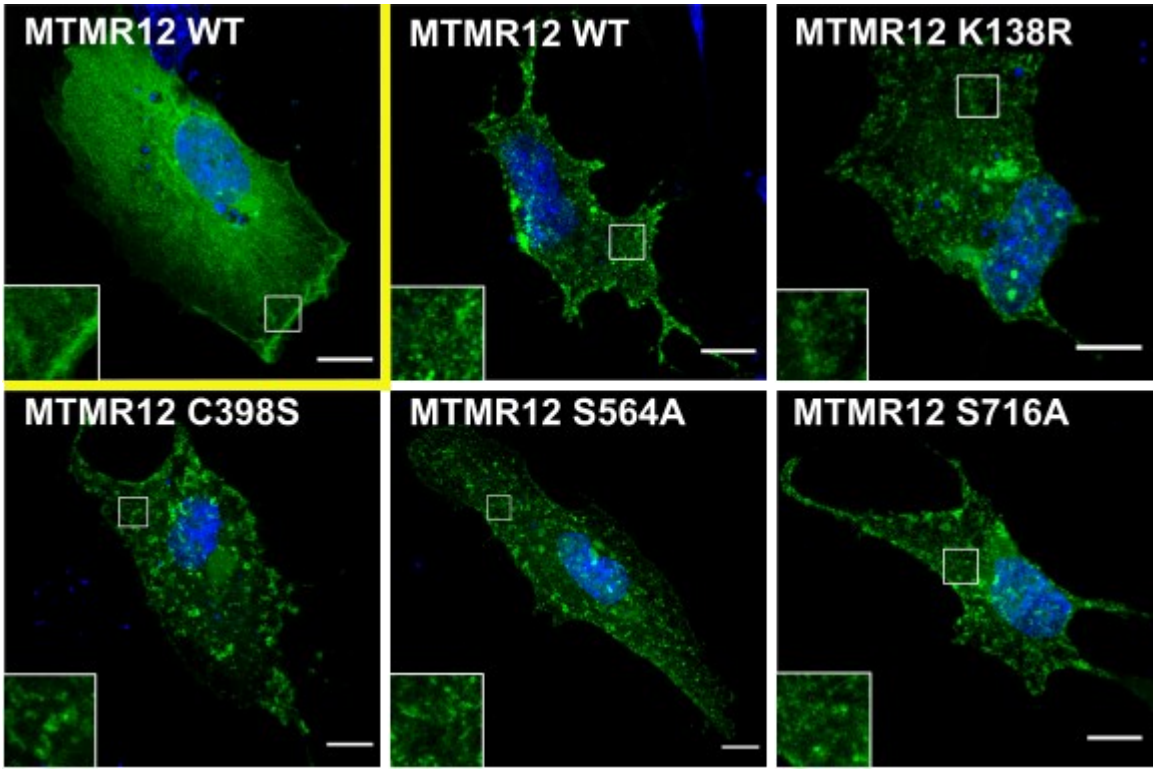


Figure 11: ***MTMR12 re-localizes into undefined vesicle-like structures upon starvation, independently of selected posttranslational modifications***. RPE-1 were transfected with MTMR12-EGFP variants and starved for 4 hours before fixation. Non-starving control is shown in the yellow square. MTMR12 protein was detected using EGFP-tag; Scale bar: 10 μm.

Since the process of autophagosome initiation is situated to the membrane of the ER, we also employed the ER protein marker (calreticulin-KDEL) and co-expressed it with MTMR12-EGFP. The co-localization between the ER marker and MTMR12-EGFP was notable (**Fig. 12**). These results suggest that MTMR12-EGFP partially localizes into sites of autophagosome formation upon starvation. Since only partial overlap with the autophagy markers was observed, we extended our analyses and used also markers for other compartments. Based on previous reports describing the roles of MTMRs in the endosomal transport, we tested if any of the MTMR12-EGFP structures are related to the endosomal pathway (Tsujita et al. 2004; Cao et al. 2008; Naughtin et al. 2010). We used markers for early endosome (EEA1), late endosome (RAB7), recycling endosome (RAB11) and lysosome (LAMP1) for co-localization analyses. However, no remarkable association was detected (**Fig. 12**). We came to a conclusion that MTMR12-EGFP partially localizes into the sites of autophagosome initiation upon starvation. However, larger set of cells needs to be measured in the future to confirm this hypothesis.

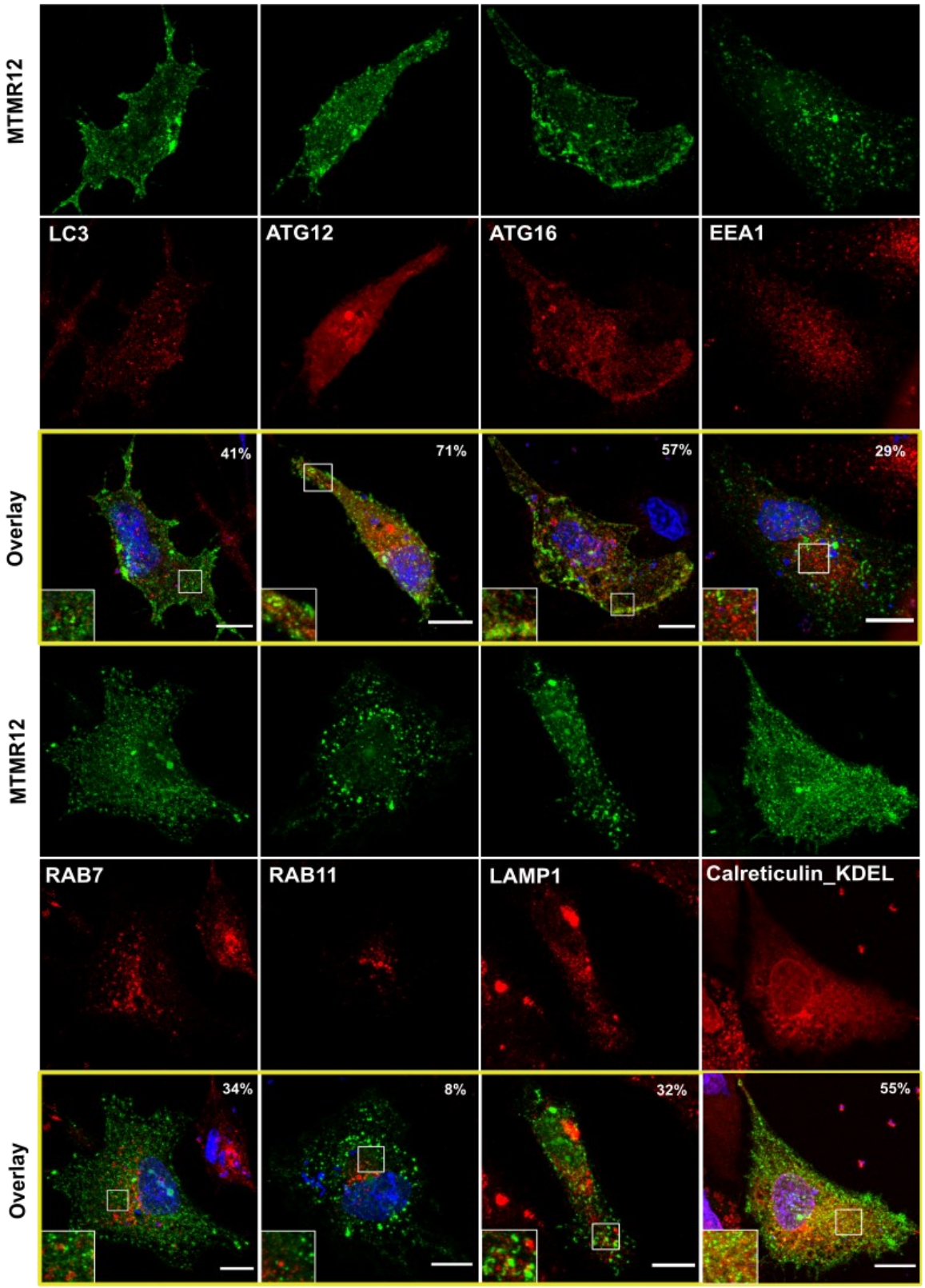


Figure 12: ***MTMR12-EGFP co-localizes with early autophagic markers upon induction of starvation*****.** RPE-1 cells were starved for 4 hours before staining. MTMR12-EGFP re-localization pattern was related to markers for autophagosome (LC3B), early autophagosome (ATG12; ATG16), EE (EEA1), LE (RAB7); recycling endosome (RAB11), lysosome (LAMP1) and ER (Calreticulin/KDEL). MTMR12-EGFP evinced the highest overlap with early autophagic and ER markers. Overley is highlighted with the yellow lines. The rate of co-localization is drawn with Pearson's correlation coefficient in the top right corner of each overlay picture. Scale bar: 10 μm.

3.4. MTMR12 co-localizes with the active member MTM1 in the cytoplasm and at the plasmatic membrane independently of the selected posttranslational modifications

Another tempting question was whether the inactive phosphatase MTMR12 influences the localization of its putative hetero-dimerization partners, the active phosphatases MTM1 and MTMR2. For this purpose, MTM1 and MTMR2 sequences were amplified from cDNA obtained from SHS cells and cloned into pcDNA4.0TOMycHis vectors with Gibson assembly

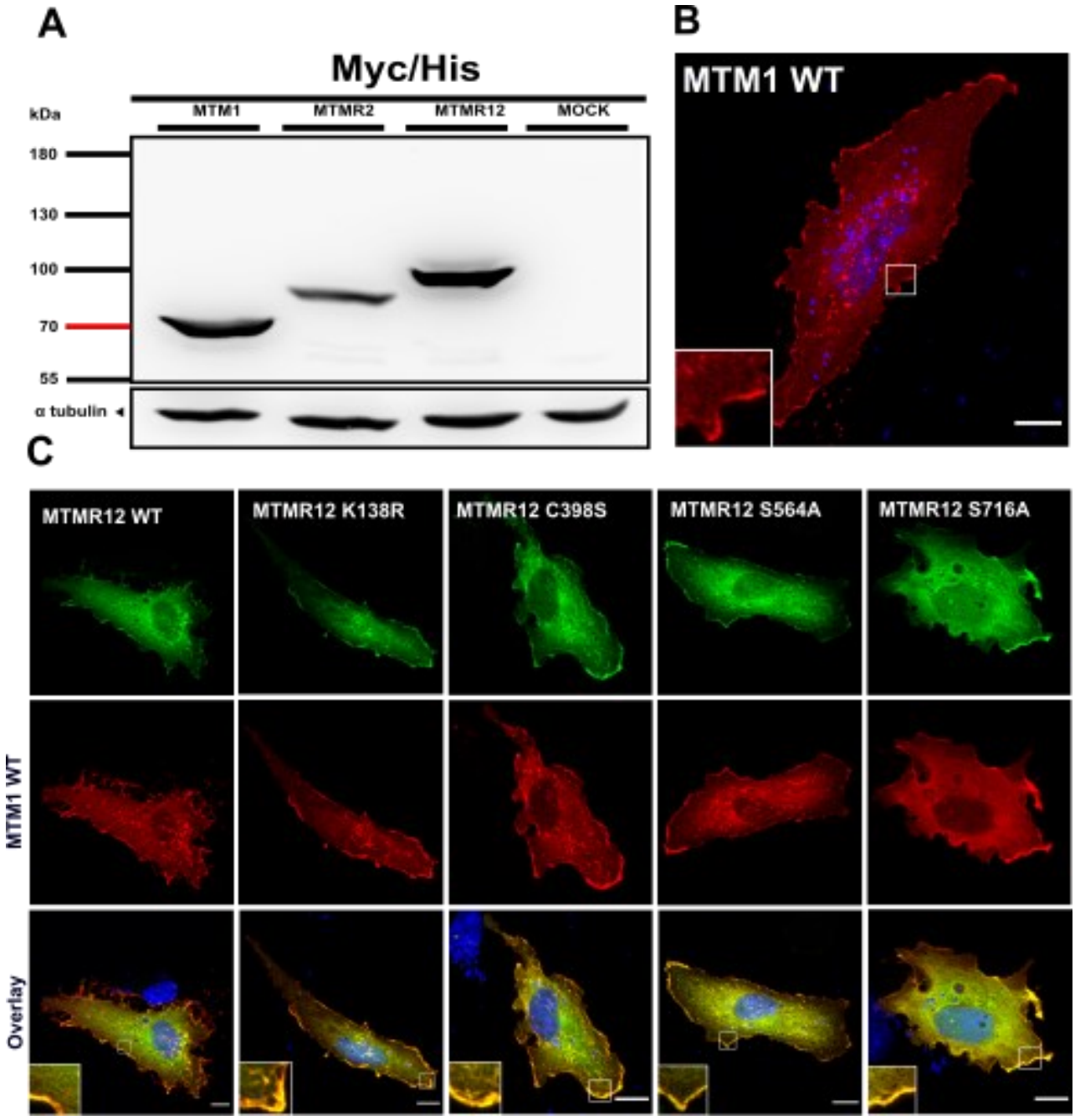


Figure 13: ***Co-expressed MTMR12 and MTM1 associate in the cytoplasm and at the plasmatic membrane independently of selected posttranslational modifications.*** **A)** MTM1-MycHis (70 kDa), MTMR2-MycHis (74 kDa) and MTMR12-MycHis (87 kDa) are being produced in the HEK293 cells. α-tubulin (50 kDa) was stained as a control. **B)** Overexpressed MTM1-MyHis localizes to the cell cytoplasm and at the PM. **C)** Co-expressed MTM1-MycHis and MTMR12-EGFP co-localize in the cell cytoplasm and at the PM. Selected posttranslational modifications do not change MTM1/MTMR12 co-localization pattern. Scale bar: 10 μm.

approach. Protein expression of MTM1-MycHis and MTMR2-MycHis was verified by Western blot approach (**Fig. 13A**).

To uncover possible relationship between localization of MTMR12 and MTM1 in the cell, MTM1/pcDNA4.0TOMycHis vector was initially transfected into the RPE-1 cells alone. Using confocal microscopy, the MTM1-MycHis protein was detected predominantly in the cytoplasm and at the PM (**Fig. 13B**). Then MTMR12-EGFP and MTM1-MycHis were overexpressed in RPE-1 cells together. As shown in **Fig. 13C**, both proteins co-localize to the same structures at the PM and in the cytoplasm, copying the pattern of both proteins overexpressed individually (**Fig. 9; Fig. 13B**). We then wanted to explore whether selected posttranslational modifications of MTMR12 affect its association with MTM1 in the cell. Co-expression of MTMR12-EGFP mutants with MTM1-MycHis resulted in the same co-localization pattern as seen with WT MTMR12-EGFP indicating that the selected posttranslational modifications do not influence MTMR12-MTM1 co-localization (**Fig. 13C**). To summarize, MTM1 and MTMR12 associate with the same structures in the cell (the PM and the cytoplasm) and do not induce any obvious mutual re-localization under the standard conditions. The second part of the experiment showed that selected posttranslational modifications of MTMR12 have no impact on this co-localization.

3.5. MTMR12 co-localizes with the active member MTMR2 in the cytoplasm, the Golgi apparatus and at the plasmatic membrane independently of the selected posttranslational modifications

To describe the relationship between MTMR12 and MTMR2, we repeated the same set of experiments as for MTM1 and MTMR12. Firstly, we individually overexpressed MTMR2-MycHis in RPE-1 cells followed by confocal microscopy analysis. MTMR2 was distributed through the cell cytoplasm, at the PM and the Golgi apparatus (GA) (**Fig. 14A**). Secondly, MTMR12-EGFP was then co-expressed with MTMR2-MycHis in RPE-1 cells. Both proteins co-localized partly in the structures, characteristic for both proteins (PM and cytoplasm) and

partly in the GA, which is rather typical for MTMR2 (**Fig. 9; Fig. 14A, B**). We then tested if selected posttranslational modifications of MTMR12 affect the co-localization between MTMR12 and MTMR2. However, overexpression of MTMR12-EGFP mutants with MTMR2-MycHis resulted into the same outcomes as seen for WT MTMR12-EGFP (**Fig. 14B**). This implicate that selected posttranslational modifications of MTMR12 do not influence MTMR2/MTMR12 association. To sum up, these results suggest that MTMR2 and MTMR12 associate to the same structures in the cell (cytoplasm, PM and GA). It is presumable that MTMR2 facilitates localization of MTMR12 to the GA. The data also implicate that selected posttranslational modifications of MTMR12 have no impact on these observations.

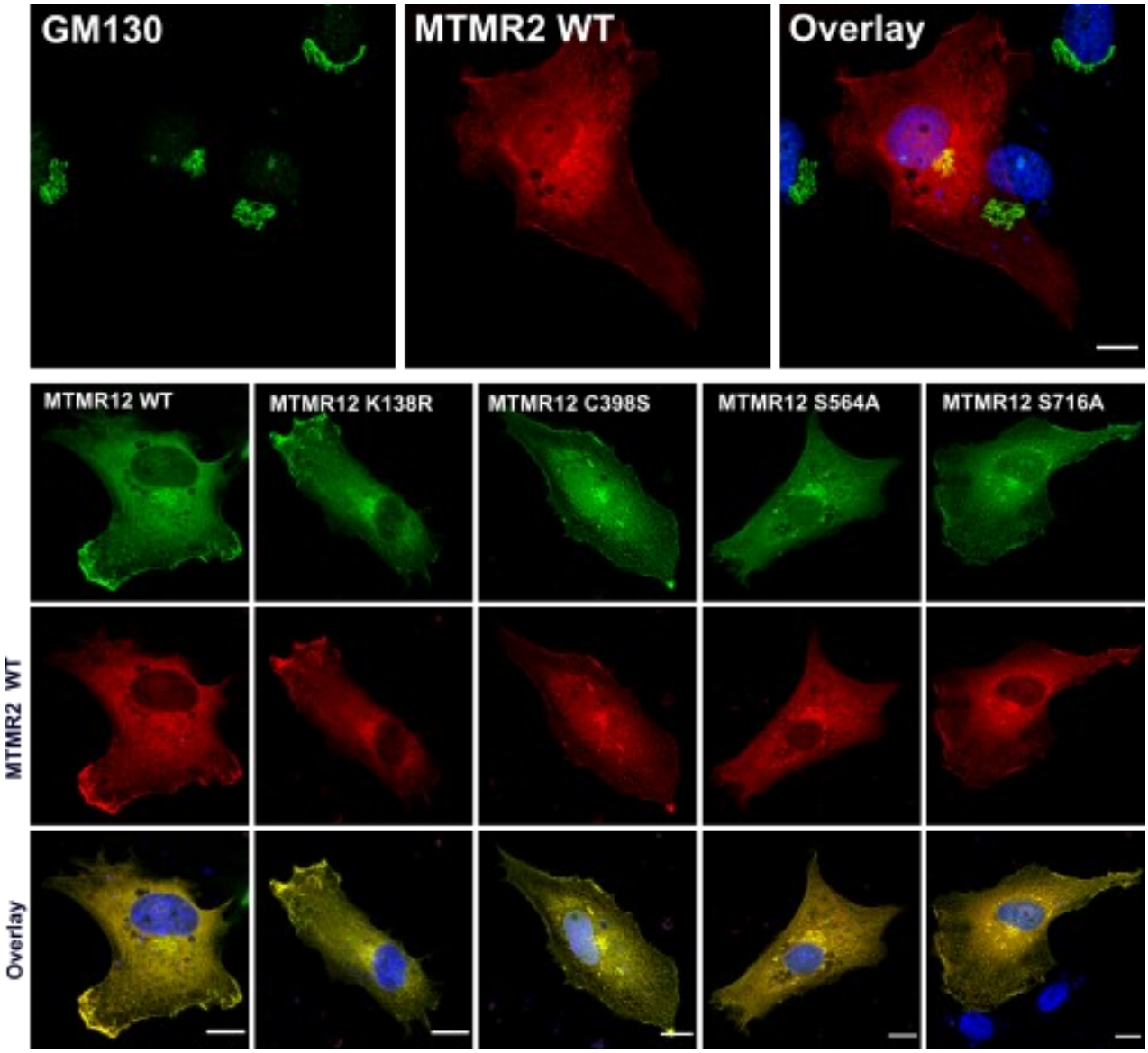
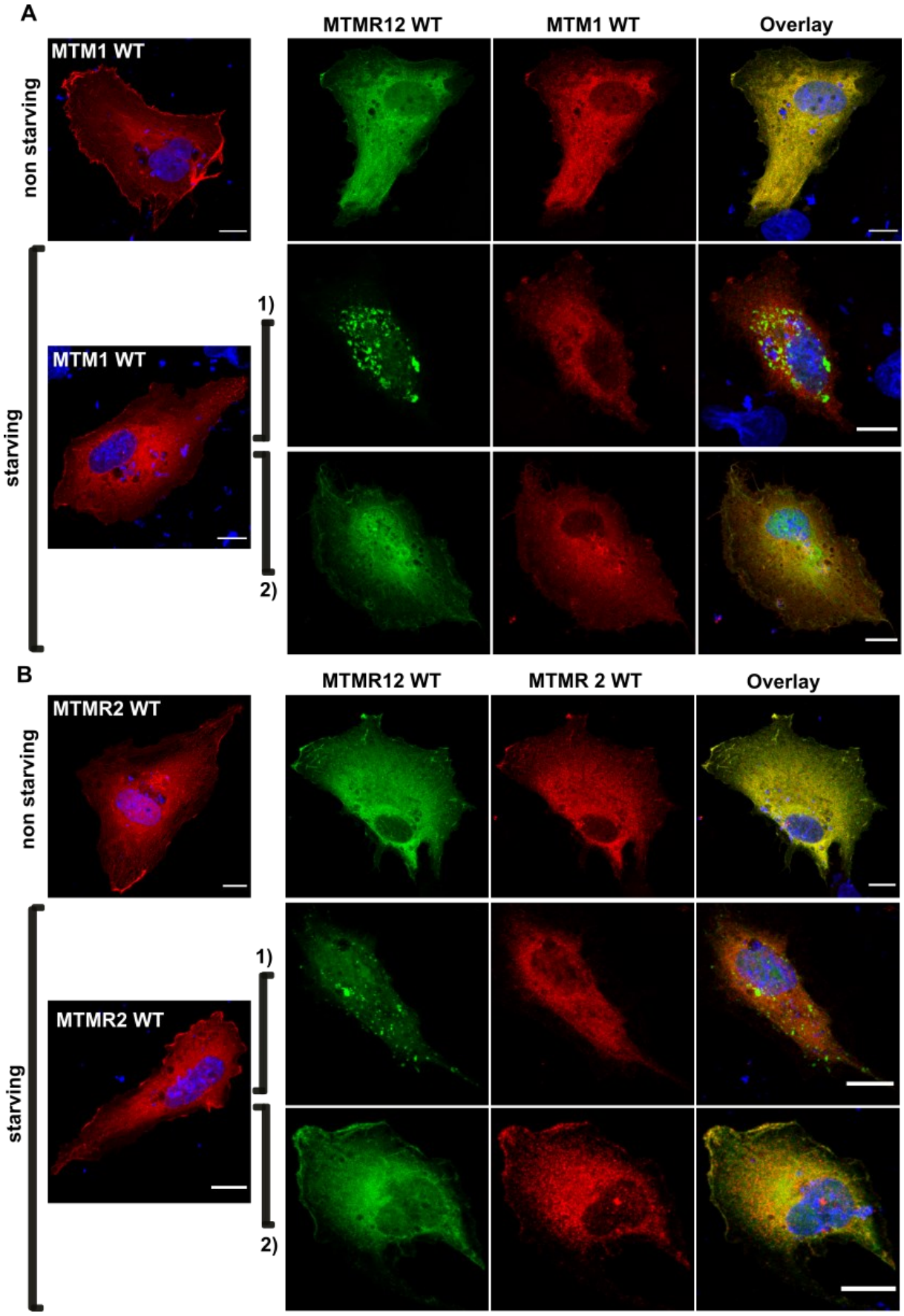


Figure 14: ***Co-expressed MTMR12 and MTMR2 associate in the cytoplasm, on the Golgi apparatus and at the plasmatic membrane independently of selected posttranslational modifications.*** **A)** Overexpressed MTMR2-MycHis localizes to the cell cytoplasm, in the cis-GA (GM130) and at the PM. **B)** Co-expressed MTMR2-MycHis and MTMR12-EGFP co-localize in the cell cytoplasm, on the GA and at the PM. Selected posttranslational modifications do not change MTMR2/MTMR12 co-localization pattern. Scale bar: 10 μm.

3.6. Starvation of the cells overexpressing MTM1/MTMR12 or MTMR2/MTMR12 complex results in two different localization phenotypes

Based on our previous observation that MTMR12 re-localizes to the vesicle-like structures upon starvation (**Fig. 11**), our last aim was to explore whether induction of starvation affects co-localization of MTMR12 with its active partners MTM1 and MTMR2. Firstly, overexpressed MTM1-MycHis and MTMR2-MycHis were observed alone upon starvation. Distribution of both of these proteins within the cell remained the same when the RPE-1 cells were starved compared to the non-treated cells (**Fig. 15 A, B**). As a next step, both MTM1-MycHis and MTMR2-MycHis were co-expressed with MTMR12 WT in RPE-1 cells. Induction of autophagy resulted in two distinct phenotypes; (1) MTMR12-EGFP manifested the same starving pattern observed when starved alone (**Fig. 11; Fig. 15 A, B**), nevertheless it lost its association with the active partners MTM1-MycHis and MTMR2-MycHis. The active partners additionally seemed to partially disappear from the PM in favour of their cytoplasmic localization, although this has not been quantified (**Fig. 15 A, B**). (2) MTMR12-EGFP lost its original starving phenotype and copied the pattern of its active partners MTM1-MycHis and MTMR2-MycHis (same as for non-treated cells) (**Fig. 11; Fig. 15 A, B**). For these experiments, mutants of MTMR12 were excluded, with regards to their non-significant role in previous experiments. To conclude, localization of active members MTM1-MycHis and MTMR2-MycHis remains the same upon starvation compared to non-treated cells. When the proteins are co-expressed with MTMR12 and exposed to starvation, two phenotypes are observed. In the first phenotype active members associate with MTMR12 at the PM and in the cytoplasm and potentially repress MTMR12 intrinsic propensity to re-localize into the starvation induced structures. Alternatively, MTMR12 undergoes re-localization to undefined structures and loses its association with the active partners MTM1 and MTMR2.


A
MTMR12 WT
MTM1 WT
Overlay
non starving
MTM1 WT
starving
1)
MTM1 WT
2)
B
MTMR12 WT
MTMR 2 WT
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non starving
MTMR2 WT
starving
1)
MTMR2 WT
2)

Figure 15*: Co-expressed MTM1-MycHis or MTMR2-MycHis with MTMR12-EGFP display two distinct phenotypes in starved RPE-1 cells.* **A)** RPE-1 cells were co-transfected with MTM1-MycHis and MTMR12-EGFP and starved for 4 h. Upon starvation, the cells showed two distinct phenotypes (1 and 2) and localization of MTM1-MycHis was compared with single-expressed MTM1-MycHis. The results were related to the non-starving conditions**. B)** Same experiment was repeated for MTMR2-MycHis and MTMR12-EGFP; Scale bar: 10 μm.

4. Discussion

After more than three decades of research, new roles of MTMRs are still appearing in a wide spectrum of cellular events and new pathologies are being linked to their malfunctions. Concurrently, there is a growing effort to understand the mechanism of MTMRs function. In this context, inactive members of MTMR family are of a particular interest as key regulators of their active partners (Raess et al. 2017). MTMR10 and MTMR12 are two inactive phosphatases from the MTMR family which share their interaction partners, the active phosphatases MTM1 and MTMR2 (Raess et al. 2017). MTM1 and MTMR2 are clinically significant proteins since their mutations cause serious hereditary diseases, X-linked myotubular myopathy and CMT-4B neuropathy, respectively (Laporte et al. 1996; Bolino et al. 2000). This makes the inactive proteins MTMR10 and MTMR12 attractive for research as potential regulators of MTM1 and MTMR2. Based on the currently available sources, MTMR12 was suggested to stabilize the active member MTM1 in skeletal muscles through the formation of a hetero-complex (Gupta et al. 2013). However, the mechanism of MTMR12 function in skeletal muscles has not yet been fully unveiled. MTMR10 shares a moderate level of protein organization with MTMR12 and was also shown to interact with the active member MTM1 (Robinson and Dixon 2006). It is therefore reasonable to ask, whether MTMR10 might have a similar role in the skeletal muscles as MTMR12. Much more focus was directed in the past on the inactive members MTMR5 and MTMR13 in the context of regulation the active MTMR2. These proteins form hetero-interactions exclusively with the active member MTMR2 (Raess et al. 2017). MTMR2/MTMR5 and MTMR2/MTMR13 hetero-complexes were reported to sequentially regulate parts of PNS development (Mammel et al. 2019). It remains to be elucidated, whether MTMR10 and MTMR12 might have redundant or additional functions in the PNS development as potential interactors and regulators of the active MTMR2. Neither it was explored whether MTMR10 and MTMR12 have another role in another tissue. The current knowledge about MTMR10 and MTMR12 proteins is limited. The aim of this thesis was to explore the roles of MTMR10 and MTMR12 in mammalian cells. We approached that by overexpressing the proteins in selected human cultured cell lines and studying localization of these proteins with the confocal microscopy.

Our first aim was to define the localization of MTMR10 and MTMR12 in mammalian cells. Overexpression of MTMR10-MycHis in HeLa cells resulted in protein distribution through the cytoplasm with local enrichment at the PM (**Fig. 7**). At certain point of our research, we were

no longer able to detect expression of the transfected MTMR10-MycHis protein in HeLa cells or in HEK293 cells for an unknown reason (data not shown). Despite all the quality control checks including MTMR10 sequence verification the expressions of MTMR10-MycHis could not been successfully repeated. We therefore postponed our experiments with MTMR10 and redirected our attention to MTMR12.

It was shown previously that MTMR12 localizes into punctate structures in addition to its cytosolic staining in COS-7 cells (Nandurkar et al. 2003). Consistently, we show that overexpressed MTMR12-EGFP is distributed through the cell cytoplasm (**Fig. 9B**). However, in our results we do not observe any punctate staining, rather the MTMR12-EGFP protein localizes to parts of the PM in both HeLa and RPE-1 cells (**Supplementary fig. 1A; Fig. 9**). This discrepancy could be explained by the fact that COS-7 cell line, used by the other laboratory, was derived from monkey fibroblasts where the conditions might differ from those in our cell lines. We observed the same localization pattern when overexpressing MTMR12 with either EGFP or MycHis tag attached to the C-terminal end of the protein (data not shown), therefore we assume that the type of tag does not interfere with the localization of the protein. C-terminal end was the preferred position for the tag attachment, since N-terminally placed tags interfered with proper protein localization of other MTMRs, as previously observed in our group and by others (data not shown; Laporte et al. 2002; Zou et al. 2009).

Up to nowadays several active MTMRs were reported to regulate autophagy, potentially by controlling the level of PtdIns3P, the key mediator of autophagosome formation (Vergne and Deretic 2010; Shibutani et al. 2015). Inactive MTMRs were also described to play a role in the regulation of autophagy. For example, a hetero-complex formed from the inactive member MTMR9 and the active member MTMR8 was shown to inhibit basal autophagy, i.e. autophagy not induced by nutrient deprivation or external stimuli (Zou et al. 2012). Consistently, the experiments with KO MTMR9 cells revealed an elevation of basal autophagy (Doubravská et al. 2019). Our results show that after starvation-induced re-localization, MTMR12-EGFP signal partially overlaps with signals for the ER and autophagy related proteins ATG12 and ATG16 (**Fig. 12**). These locations could represent the sites of autophagosome initiation. MTMR12-EGFP also associates with LC3B molecule but to a lesser extent then with ATG12 and ATG16 (**Fig. 12**). LC3B appears at the isolation membrane of the emerging autophagosome after the initiation of autophagosome formation (Shibutani et al. 2015). We hypothesise that MTMR12-EGFP might have a role in the initial steps of autophagy, however it is not yet clear what is the exact role of MTMR12 in these steps (see also below). Bigger

scale of data also needs to be collected in the future to improve the statistical significance of these results.

One of the key motivations for studying MTMR12 was its potential role in regulation of the active partners MTM1 and MTMR2. To be able to examine putative relationship between these members, we first wanted to explore localization of MTM1 and MTMR2 in the absence of MTMR12. We started with localization of MTM1-MycHis and MTMR2-MycHis in RPE-1. We show in **Fig. 13B** and **Fig. 14A** that overexpression of these proteins leads to their cytosolic distribution with partial localization to the PM. Besides, MTMR2 is additionally enriched in the GA. This observation is consistent with previous reports that MTMR2 is a cytosolic protein which evinces stronger perinuclear staining (Laporte et al. 2002; Kim et al. 2002; Berger et al. 2003; Robinson and Dixon 2006). We hypothesize that the enzymatically active MTMR2 could function at membranes of the GA to control local subpools of PtdIns3P. However, this hypothesis requires experimental confirmation. We also observe that increased transfection leads to an altered cell morphology and formation of extensive PM projections in case of MTM1-MycHis (**Supplementary fig. 2**), whereas in case of MTMR2, the cell morphology remains the same (data not shown). Since this phenotype is characteristic only for MTM1-MycHis and is not seen with MTMR2-MycHis, we conclude that MTM1 and MTMR2 could have different functions at the PM. Although previous studies had not been specifically pointing out MTMR2 localization at the PM, its presence there could be seen in the published images (Laporte et al. 2002; Kim et al. 2002; Berger et al. 2003). Our observations therefore do not present any conflict with the published data. On the other hand, MTM1 localization at the PM was extensively studied in the past. It was shown that MTM1 substrate-trap mutants are retained in the PM projections (Blondeau et al. 2000; Taylor et al. 2000). These projections were further described as Rac1-induced ruffles (Laporte et al. 2002). MTM1 was suggested to localize to these ruffles to perform its enzymatic activity on subpools of PtdIns3P (Laporte et al. 2002). These reports are in harmony with our observation that MTM1 accumulates at the PM, proportionately to the level of expression.

It was shown previously that the active and the inactive MTMR members associate in cells to regulate their subcellular localization. For example, inactive MTMR9 was shown to recruit the active partners MTMR6 and MTMR8 to the membranes of the intermediate compartment and the GA (Doubravská et al. 2019). We were therefore interested if the localization of active MTM1 and MTMR2 is altered in the presence of MTMR12. For that we performed series of co-localization experiments. Firstly, we co-expressed MTMR12-EGFP with either MTM1-

MycHis or MTMR2-MycHis in RPE-1 cells. In both cases, MTMR12 co-localized with MTM1 and MTMR2 in the cytoplasm and at the PM (**Fig. 13C; Fig. 14B**). Moreover, MTMR12 staining overlapped with MTMR2-MycHis at the GA (**Fig. 14B**). We hypothesize that MTMR2 recruits MTMR12 to the membranes of the GA, where MTMR12 could function to either stabilize MTMR2 or regulate MTMR2 activity. Alternatively, MTMR12 might be recruited to the membranes of GA by MTMR2 to play an independent role, possibly as a scaffold molecule for other proteins. Regarding the co-localization on the PM, it was reported previously that MTMR12 removes its active partner MTM1 from the PM to the cytosol (Nandurkar et al. 2003). We cannot see this tendency in our results, possibly due to the use of a different model cell line. It is also possible that the mutual interaction is influenced by the level of expression of each of the partner. Based on our results, it would be interesting to test whether one of the associating members MTMR12 or MTM1/MTMR2 has a dominant role in recruiting its partner to the PM. Fractionation of individually transfected cells and cells co-transfected with MTMR12 and either MTM1 or MTMR2 could be performed to test that. Direct hetero-interactions were previously described between various MTMRs (Raess et al. 2017). Strong co-localization between MTMR12-EGFP and MTM1-MycHis or MTMR2-MycHis suggests that overexpressed MTMR12-EGFP physically interacts with the active partners in RPE-1 cells (**Fig. 13C; Fig. 14B**). Reciprocal co-immunoprecipitation of co-expressed MTMR12-EGFP with the active partners MTM1-MycHis and MTMR2-MycHis would help to verify this hypothesis.

Induction of starvation resulted into re-localization of individually transfected MTMR12-EGFP into vesicle-like structures, mostly positive for the ATG12 autophagic marker (**Fig. 12**). Our last aim was to identify, whether the co-localization between MTMR12-EGFP and the active partners MTM1-MycHis and MTMR2-MycHis is affected during starvation-induced autophagy. As seen in **Fig. 15 A, B** induction of starvation does not result in change in localization of individually expressed MTM1-MycHis or MTMR2-MycHis. However, when starving the cells with co-expressed MTMR12-EGFP and MTM1-MycHis or MTMR2-MycHis, we could see two distinct phenotypes. I) MTMR12-EGFP lost its association with the active partners and re-localized into the same vesicle-like structures as observed when expressed and starved alone (**Fig. 11; Fig. 15 A, B**). In cells displaying this phenotype, the active members appear to preserve their cytoplasmic localization and partially decrease their localization at the PM. However, quantification of the fluorescent signal needs to be done in the future experiments to confirm this observation. This phenotype suggests that MTMR12

might have a separate function independent of MTM1 and MTMR2 on vesicle-like structures, possibly acting as a scaffold protein at the site of autophagosome initiation. II) The second phenotype shows an identical co-localization pattern to that observed in non-starving cells (**Fig. 11; Fig. 15 A, B**). This could mean that the active members MTM1 and MTMR2 negatively regulate MTMR12 by preventing MTMR12 from re-localization into starvation-induced vesicle-like structures observed for individually transfected MTMR12-EGFP. We provide two possible explanations for the parallel occurrence of these two phenotypes. First option is that the active members MTM1 and MTMR2 act during the initial stages of nutrient deprivation to hold MTMR12 in the cytoplasm. When the nutrient deprivation continues, MTMR12 dissociates from the active members and re-localize into the vesicle-like structures. Alternative explanation is that the second observed phenotype is represented by the cells where autophagy was not yet induced. Individual cells might respond to nutrient deprivation with a variable sensitivity. For that reason, it would be meaningful to repeat the same set of experiments using rapamycin for induction of autophagy. Rapamycin is a direct inhibitor of the rapamycin complex 1 (mTORC1), a master regulator of autophagy. mTORC1 integrates various nutritional signals and its inhibition by rapamycin appears to be essential for initiation of autophagy (Dossou and Basu 2019). Also, a larger set of samples needs to be measured in future to support the data with statistics.

The puzzling property of MTMR proteins is that they are commonly detected in the cytoplasm, while their enzymatic function is required at various cellular membranes (Laporte et al. 2002; Lorenzo et al. 2006, our own observations). It is probable that they localize to the membranes only transiently. Subcellular localization of proteins can be modulated by posttranslational modifications. It was reported previously that MTMR2 changes its localization upon phosphorylation (Franklin et al. 2013). It is therefore presumable that other MTMR members can be regulated similarly. We were interested if also localization of MTMR12 and its association with the active members is affected by any modification. Initial hint that indeed some posttranslational modification might be present on MTMR12 came from the observation of an additional band of MTMR12-EGFP and MTMR12-MycHis proteins detected by Western blot after expression in HEK293 cells. (**Supplementary fig. 1B; Fig. 13A**). To explore that, we selected four potentially interesting modifications for MTMR12, as seen in **Fig. 10,** based on predictions from the software tool CSS-Palm (csspalm.biocuckoo.org) and mass spectrometry data found in the PhosphoSitePlus database (phosphosite.org). We were particularly interested in three types of modifications – phosphorylation, ubiquitination and

palmitoylation. All these modifications are reversible, therefore have potential to regulate the protein's behaviour in response to intracellular or extracellular signal (Charollais and Van Der Goot 2009). Phosphorylation and ubiquitination are among the most prevalent modifications modulating the stability, activity and localization of the proteins in the cell (Cheng et al. 2011; Swatek and Komander 2016). Besides, MTMR2 was observed to change its localization upon phosphorylation of two aa by MAPK/Erk1, thereby phosphorylation appears as a highly presumable mechanism regulating other MTMR members (Franklin et al. 2013). Palmitoylation represents a type of lipidic covalent modification. It is being commonly found on soluble proteins where it mediates transient interactions with membranes (Jiang et al. 2018). We show in **Fig. 9B** that disruption of selected modifications by mutation of corresponding aa does not induce re-localization of MTMR12-EGFP protein upon standard conditions. However, we worked on the assumption that the selected modifications are being present in the WT MTMR12 in RPE-1 cells and that directed mutagenesis disrupts their effect. Before making a definitive conclusion about the role of the modified sites it would be correct to confirm the modification status of these sites in MTMR12-EGFP by our own mass spectrometry data. Reversible modifications are often created in response to some extracellular signal. Particular signal can lead to different cellular conditions under which posttranslational modifications can be potentially created. We approached that by stimulating starvation-induced autophagy. Upon starvation, we observed re-localization of WT MTMR12-EGFP into undefined punctate structures (**Fig. 11**). Since MTMR12 mutants showed the same localization pattern, we speculate that selected MTMR12 posttranslational modifications do not play an important role in this process (**Fig. 11**). MTMR2 re-localization was shown to be regulated by MAPK/Erk1 dependent phosphorylation (Franklin et al. 2013). Since MAPK/Erk1 activity can be induced by growth factor stimulants, it would be therefore interesting to further examine the selected posttranslational modifications of MTMR12 upon stimulation of EGFR (Guo et al. 2020).

Based on our observations we can state that MTMR12 associates with MTM1 and MTMR2 in the cytosol, at the PM and in case of MTMR2 also in the GA (**Fig. 13C; Fig. 14B**). Comparing the protein distribution of MTM1 or MTMR2 co-expressed with either WT MTMR12 or its mutated version, we observed no change in the localization pattern (**Fig. 13C; Fig. 14B**). We conclude that selected posttranslational modifications do not affect the association of MTMR12 with the active partners MTM1 and MTMR2 upon standard conditions.

Our experiments brought some information about the behaviour of MTMR12, but there are still many questions unanswered and there are many directions in which the future analyses of the inactive MTMRs could proceed. It was shown for the active MTMR2 and inactive MTMR13 that these proteins form a hetero-tetramer complex, preceded by MTMR2 and MTMR13 homodimerization (Berger et al. 2006). MTMR2 and MTMR13 homodimerization was seen to be dependent on the presence of CC domains and was suggested to increase MTMRs affinity towards membranes (Berger et al. 2006). Disruption of CC domains not only impaired the homo-interactions and subsequent hetero-interactions, but also led to an altered distribution of MTMR2 protein (Berger et al. 2003). It would be therefore interesting to examine, whether the CC domain of MTMR12 mediates its putative homo-dimer interaction. Simultaneously, it would be potentially interesting to explore which domains of MTMR12 are responsible for the subsequent interactions with the active members MTM1 and MTMR2 and which domains are responsible for MTMR12 binding to the membranes. Based on the observations of other MTMRs, PH-GRAM domain was shown to bind to various PIPs and other lipids and to mediate the membrane interaction; therefore it is an attractive candidate for examination (Tsujita et al. 2004; Choudhury et al. 2006; Berger et al. 2006).

MTMRs behaviour appears to be responsive to the conditions where their substrates levels are changed such as induction of autophagy. We examined the behaviour of MTMR12-EGFP upon the starvation-induced autophagy and observed re-localization of MTMR12-EGFP into vesicle like structures (**Fig. 11).** MTMRs were previously described to change their localization in response to other cellular conditions. As an example, MTM1 and MTMR2 were seen to re-localize from cytoplasm to the endosomal structures upon stimulation of EGFR to regulate the endosomal-lysosomal pathway (Tsujita et al. 2004; Cao et al. 2008). Similarly, MTMR2 was reported to re-localize to the membranes of large vacuoles formed upon the hypoosmotic shock (Berger et al. 2003). Interestingly, co-expression of MTMR13 with MTMR2 upon hypoosmotic conditions showed that MTMR13 preferentially binds to the membranes of formed vacuoles. This suggests that MTMR13 blocks MTMR2 function at the vacuolar membranes upon these conditions by competing for membrane-binding sites (Berger et al. 2006). Further characterization of MTMR12 localization and association with active partners upon other conditions such as induction of EGFR or hypoosmotic shock would shed more light on the function of this protein. Additional employment of the life-cell imaging would further enable to observe the behaviour of MTMRs upon induction of different conditions in the real time.

The expression profiles of MTMR proteins and their isoforms were partially mapped (Raess et al. 2017). We attempted to isolate all the protein-coding isoforms of MTMR10 and MTMR12 from SHS and RPE cells. However, only the most canonical isoforms were obtained, possibly due to the low expression levels or poor stability of other isoforms in these cell types We suggest that characterization of MTMR12 and MTMR10 proteins and their isoforms in the most relevant tissues could help uncover their functions.

5. Conclusion

Members of the MTMR family represent a perspective field for studying due to their irreplaceable role in the regulation of the key cellular processes such as endosomal transport and autophagy. We aimed to explore the behaviour of inactive myotubularins MTMR10 and MTMR12 in the cultured mammalian cells. We found that both MTMR10 and MTMR12 are dispersed through the cytoplasm of mammalian culture cells with partial enrichment at the PM. Co-expression of MTMR12 with the active members led to the strong co-localization with no effect of the active MTMRs. Reversely, MTMR2 appeared to recruit MTMR12 at the membranes of Golgi apparatus. We show that selected posttranslational modifications of MTMR12 have no effect on the localization of MTMR12 upon standard conditions. Interestingly, stimulation of starvation induced autophagy led to re-localization of MTMR12 into undefined structures, mostly overlapping with early autophagosomal marker ATG12. This MTMR12 specific phenotype was partially observed also upon starvation of cells with co-expressed MTMR12 with MTM1/MTMR2. This phenotype was connected with the disruption of MTMR12 and MTM1/MTMR2 co-localization suggesting that MTMR12 could have an MTM1/MTMR2 independent function at the undefined structures upon induction of autophagy. More experiments need to be done in the future to understand the various roles of MTMR10 and MTMR12 in the cell and organism.

6. Materials & methods

6.1. Materials

6.1.1. Buffers and solutions

Lysogeny broth (LH) medium

For 200 ml of LB:

- 2 g bacto tryptone
- 2 g yeast extract
- 2 g sodium chloride (NaCl)

Mixed and autoclaved. Stored at 4°C.

Agar plates for bacterial culture growth

- LB mix with agar – 35 g/ 1l ddH_2O (Sigma-Aldrich)

Mixed and autoclaved. When cooled at 60°C the mixture was supplemented with antibiotics – ampicillin (AMP, 50 mg/ml, diluted 1:500) / kanamycin (KAN, 25 mg/ml, diluted 1:1000) – and poured on plates.

Tris-acetate-EDTA (TAE) buffer

- 40 mM Tris (pH 8.0)
- 20 mM acetic acid ($C_2H_4O_2$)
- 1 mM ethylenediaminetetraacetic acid (EDTA)

Tris-borate-EDTA (TBE) buffer

- 89 mM Tris (pH 8.0)
- 89 mM boric acid (H3BO3)
- 2 mM EDTA

5x ISO buffer

- 0.5 M Tris-HCl (pH 7.5)
- 50 mM magnesium chloride ($MgCl_2$)
- 4 mM dNTP mix (1 mM each)
- 50 mM dithiotreitol (DTT)
- 31 mM (PEG)-8000 ($H(OCH_2CH_2)nOH$)
- 5 mM β -nicotinamide adenine dinucleotide hydrate (NAD)

Stored at –20°C in 320 µl aliquots.

Gibson assembly master mix

- 26,6% (w/v) 5x ISO Buffer
- 6 U T5 exonuclease
- 40 U Phusion polymerase
- 6400 U Taq ligase

Stored at –20°C in 15 µl aliquots.

Alkaline lysis mini prep solutions

Solution 1

- 25 mM Tris (pH 8.0)
- 10 mM EDTA (pH 8.0)
- 50 mM glucose ($C_6H_{12}O_6$)
- ddH_2O to 100 ml

RNase added 100 µg/ml, stored at 4 °C.

Solution 2

- 1% (w/v) sodium dodecyl sulfate (SDS)
- 200 mM NaOH

Stored at room temperature.

Solution 3

- 3 M potassium acetate (CH_3CO_2K)
- 11,46 % (w/w) glacial acetic acid

Stored at 4°C.

Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific)

DMEM++

DMEM supplied with:

- 10% fetal bovine serum (FBS)
- 100 U/ml of penicillin and 100 µg/ml of streptomycin

Phosphate buffer saline (PBS)

- 197 mM NaCl
- 2.7 mM potassium chloride (KCl)
- 10 mM disodium phosphate (Na_2HPO_4)
- 1.8 mM monopotassium phosphate (KH_2PO_4)

Premixed as 10 x solution. pH adjusted to 7.4.

Hank's Buffered Salt Solution (HBSS) (Thermo Fisher Scientific)

Laemmli Sample Buffer 4x

- 200 uM Tris(hydroxymethyl)aminomethan (Tris) (pH 6.8)
- 0.16 % (w/v) SDS
- 4% (w/v) glycerol ($C_3H_8O_3$)
- 1% (w/v) mercaptoethanol (C_2H_6OS)
- 0.02 % (w/v) bromphenol blue

Stored aliquots at 20°C.

Blotting buffer (Towbin)

- 25 mM Tris
- 192 mM glycine
- 20% (w/v) methanol

Filtered and stored at 4°C.

Running buffer

- 25 mM Tris base
- 192 mM glycine ($C_2H_5NO_2$)
- 0.1 % (w/v) SDS

Filtered and stored at room temperature.

Separation polyacrylamide gel (9%)

- 375 mM Tris (pH 8.8)
- 9% (w/v) Acrylamide/bis-acrylamide mix 29:1 (Sigma-Aldrich)
- 1% SDS (w/v)
- 0,05% (w/v) ammonium persulfate (APS)
- 0,04% (w/v) tetramethylethyletediamine (TEMED)

Stacking polyacrylamide gel (3,9%)

- 125 mM Tris (pH 6.8)
- 3,9% (w/v) Acrylamide/bis-acrylamide mix 29:1
- 0,1% (w/v) SDS
- 0,12% (w/v) APS
- 0,8% (w/v) TEMED

TBST buffer

- 10 mM TRIS-Cl
- 150 mM NaCl
- 0.05% Tween 20

pH adjusted to 7.4. Stored at room temperature.

Microtubule stabilising buffer (MSB)

- 20 mM 2-(N-morpholino)-ethanesulfonic acid (MES)
- 2 mM ethylene glycol tetraacetic acid (EGTA)
- 2 mM magnesium chloride ($MgCl_2$)
- 4% (w/v) polyethylene glycol 6000 (PEG)

3% paraformaldehyde (Pfa)

(Working in ventilated hood)

- 3 % PfA
- NaOH – added dropwise until PFA dissolves (clear solution)

Cooled, filtered and pH adjusted to 6.9.

- 50 % 2x MSB

Stored at – 20 °C.

Mowiol mounting medium

- 3 g glycerol
- 2.4 g Mowiol 4-88 (Hoechs)
- 6 ml ddH2O
- 12 ml 0.2M Tris-HCl (pH 8.5)

Aliquotes stored at -20 °C.

6.1.2. Antibodies

Table 1: Primary antibodies

Name:	Host:	Manofacturer:	Cataloge number:	Dilution for IF:	Dilution for WB
Myc	Rabbit	Genetex/Exbio	GTX115046	1:1500	-
Myc (9E10)	Mouse	Exbio	11-433-C100	1:800	1:2000
EGFP	Mouse	Roche	11814460001	-	1:400
LC3B	Rabbit	Abcam	ab192890	1 µg/ml	1:2000
α-tubulin(DM1A)	Mouse	Sigma	T9026	1:500	Up to 1:10000
Atg12 (D88H11)	Rabbit	CST	4180	1:100	-
Atg16L1 (D6D5)	Rabbit	CST	8089	1:100	-
EEA1 (C45B10)	Rabbit	Cell Signaling	3288	1:800	-
Rab11a	Rabbit	Abcam	ab128913	1:10-100x	-
Rab7 (D95F2)	Rabbit	Cell Signaling	9367	1:300	-
LAMP-1 (D2D11)	Rabbit	Cell Signaling	9091	1:300	-
GM130	Rabbit	Abcam	ab52649	1:100-1000x	1:1000-10000

Table 2: Secondary antibodies

Name:	Manofacturer:	Catalogue number:	Dilution for IF:	Dilution for WB
GAM/HRP	Jackson Immunores./Spinchem	115-035-146	-	Up to 1:10000
DAM/Cy5	Jackson Immunores./Spinchem	715-175-150	1:400	-
GAM/A488	ThermoFisher Scientific	A11001	1:1000	-
DAR/Cy5	Jackson Immunores./Spinchem	711-175-152	1:500	
DAR/A488	Jackson Immunores./Spinchem	711-545-152	1:500	-

6.1.3. Primers

Table 3: *MTMR10* primers

Name:	Sequence:	Restriction site:	Type:
MTMR10-cDNA1_F	AAGCTTATGTTCTCCCTCAAGCCGC	HindIII	Cloning
MTMR10-cDNA1_R	CTCGAGGTCTTCATTTGCTAATGTCTCAG	XhoI	Cloning
MTMR10seq1_F	AGCCAACAGACCTCCAGC		Sequencing
MTMR10seq2_F	GCAAACATCTCTCTGTAGTCC		Sequencing
MTMR10_C411S_F	GAAGGAAGAGACTTGAGCAGTTGTGTAGCTTCTCTTG		Mutagenesis
MTMR10_C411S_R	CAAGAGAAGCTACACAACTGCTCAAGTCTCTTCCTTC		Mutagenesis
MTMR10_K611_K613R_F	TTCCAAGGAGAAATTCATTGATATTAAGACCAAGGCCAGATC CAGCTC		Mutagenesis
MTMR10_K611_K613R_R	GAGCTGGATCTGGCCTTGGTCTTAATATCAATGAATTTCTCCTT GGAA		Mutagenesis
MTMR10_S607A_F	CCAAGAATTACTTCCAAGGAGAAATGCATTGATATTAAAACC AAAGC		Mutagenesis
MTMR10_S607A_R	GCTTTGGTTTTAATATCAATGCATTTCTCCTTGGAAGTAATTCT TGG		Mutagenesis
MTMR10_S751A_F	GTAGGGAATCTGTGCAGACGAGCCATTTTAGGAACACCATTA AG		Mutagenesis
MTMR10_S751A_R	CTTAATGGTGTTCCTAAAATGGCTCGTCTGCACAGATTCCCTA C		Mutagenesis
MTMR10_Y708A_F	CCTGGAGGCCTGCGCTGGGGAGCTGGGC		Mutagenesis
MTMR10_Y708A_R	GCCCAGCTCCCCAGCGCAGGCCTCCAGG		Mutagenesis

Table 4: *MTMR12* primers

Name:	Sequence:	Restriction site:	Type:
MTMR12_Xho_F	ATACTCGAGATGCTGGGGAAAGGAG	XhoI	Cloning
MTMR12_Xho_R	AGACTCGAGCACATCCCCTAGGTC	XhoI	Cloning
MTMR12_seq1_F	AAGCAGTGAGTGTCAACG		Sequencing
MTMR12_seq2_F	ATCTGCTCACCGTGTGGG		Sequencing
MTMR12_K138R_F	GGACAACTGAAGAAATACCCTGAGAGGCTCATCATCCAC		Mutagenesis
MTMR12_K138R_R	GTGGATGATGAGCCTCTCAGGGTATTTCTTCAGTTGTCC		Mutagenesis
MTMR12_C398S_F	GAATGCATCCGACCTCAGCTGTCTCATTTCCTC		Mutagenesis
MTMR12_C398S_R	GAGGAAATGAGACAGCTGAGGTCGGATGCATTC		Mutagenesis
MTMR12_S564A_F	CTTCAAACATCAACGACAACTTGCTTTGCCACTTACACAATCTAA		Mutagenesis
MTMR12_S564A_R	TTAGATTGTGTAAGTGGCAAAGCAAGTTGTCGTTGATGTTTGAAG		Mutagenesis
MTMR12_S716A_F	CTCCAGCGACATTCCGCTAAGCCCGTCTTAC		Mutagenesis
MTMR12_S716A_R	GTAAGACGGGCTTAGCGGAATGTCGCTGGAG		Mutagenesis
MTMR12_MycHis_F	CGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCATGCTGGGGAAAG GAGTAGTCGGC		Gibson cloning
MTMR12_MycHis_R	ATGAGTTTTTGTTCGAAGGGCCCTCTAGACTCGAGCACATCCCCTAGG TCCACGAACTC		Gibson cloning

Table 5: *MTM1* primers

Name:	Sequence:	Type:
MTM1_MycHis_F	CGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCATGGCTTCTGCATC AACTTCTAAATATAATTC	Gibson cloning
MTM1_MycHis_R	ATGAGTTTTTGTTCGAAGGGCCCTCTAGACTCGAGGAAGTGAGTTTGCA CATGGGGCATC	Gibson cloning
MTM1_pEGFPN3_F	AGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTATGGCTTCTGCATC AACTTCTAAATATAATCC	Gibson cloning
MTM1_PEGFPN3_R	ACCATGGTGGCGATGGATCCCGGGCCCGCGGTACCGAAGTGAGTTTGC ACATGGGGCATC	Gibson cloning
MTM1_seq1	GTGCTATGAGCTCTGTGAC	Sequencing
MTM1_seq2	CTCGAATAGGTCATGGTG	Sequencing

Table 6: *MTMR2* primers

Name:	Sequence:	Type:
MTMR2_MycHis_F	CGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCATGGAGAAGAGCTC GAGCTGCGAG	Gibson cloning
MTMR2_MycHis_R	ATGAGTTTTTGTTCGAAGGGCCCTCTAGACTCGAGTACAACAGTTTGGA CAGGAGTGAC	Gibson cloning
MTMR2_pEGFPN3_F	AGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTATGGAGAAGAGCT CGAGCTGCGAG	Gibson cloning
MTMR2_pEGFPN3_R	ACCATGGTGGCGATGGATCCCGGGCCCGCGGTACCTACAACAGTTTGG ACAGGAGTGAC	Gibson cloning
MTMR2_seq1	CCTGTCTCTAATAACCTGCC	Sequencing
MTMR2_seq2	GGATACTATCGAACCATCCG	Sequencing

Table 7: Other primers

Name:	Sequence:	Type:
CMV_seq	CGCAAATGGGCGGTAGGCGTG	Sequencing

6.2. Cloning

SH-SY5Y (SHS) cell line derived from neuroblastoma bone marrow and retinal pigment epithelial (RPE-1) cell line were kindly provided by Doc. RNDr. Jiří Novotný, Dsc. (Charles University) and Libor Macůrek M.D., PhD. (IMG CAS), respectively. Total RNA was isolated from SHS and RPE-1 cells using RNeasy Mini Kit (Qiagen, Germany) and reversely transcribed into cDNA using SuperScript III Reverse Transcriptase and oligo dT primers (Life Technologies, USA) following the manufacturer's instructions. SHS cDNA (for MTMR10 and MTM1) and RPE-1 cDNA (for MTMR12 and MTMR2) were then used as a template for PCR amplification of desired open reading frames (ORF) with specific overhangs designed for subsequent restriction or Gibson cloning (Primers in **Tab. 3, 4, 5, 6**; reaction mixture and conditions in **Tab. 8, 9**).

Table 8: PCR reaction mixture

Template DNA (50-100 ng/µl)	1 µl
5x HF buffer	10 µl
F primer (10 µM)	1 µl
R primer (10 µM)	1 µl
dNTPs (10 mM)	1 µl
Phusion polymerase 2 U/µl	0.5 µl
Deionized water	Up to total volume of 50 µl

Table 9: PCR reaction conditions

Phase:	Temperature:	Time:	Repeats:
1. Primary denaturation	98°C	2 min	
2. Denaturation	98°C	10 s	33 x
3. Annealing	58 - 62°C	20 s	
4. Elongation	72°C	1 min/ 1000 bp	
5. Final elongation	72°C	5 min	

6.2.1. Restriction cloning

For preparation of the constructs "MTMR10/pcDNA4.0TOMycHis" and "MTMR12/pEGFP-N3", PCR products were first cloned into pJET vector via blunt ends using CloneJET PCR Cloning Kit (Thermo Fisher Scientific) following manufacturer's instructions. pJET vectors with inserted PCR products were transformed into bacterial culture DH5α and grown on agarose plates with AMP resistance. Colony PCR was then performed to select and verify the colonies positive for vectors with desired inserts (reaction mixture and conditions in **Tab. 9, 10**).

Table 10: Colony PCR reaction mixture

Template DNA (traces of DNA in 10µl of ddH_2O)	1 µl
10x Pfu buffer	2 µl
F primer (10 µM)	0.5 µl
R primer (10 µM)	0.5 µl
dNTPs (10 mM)	0.5 µl
Homemade Pfu polymerase	0.25 µl
Deionized water	Up to total volume of 20 µl

Selected colonies were then grown in LB medium and desired vectors were isolated from them using High-Speed plasmid mini kit (GeneAid). MTMR10/pJET and MTMR12/pJET vectors and "pcDNA4.0ToMycHis" and "pEGFP-N3" empty vectors were digested by corresponding restriction enzymes following manufacturer's instructions (Thermo Fisher Scientific/ Fermentas.) To prevent self-ligation, the empty vectors were treated with 1 µl of Shrimp Alkaline Phosphatase (SAP) (1U/µl) phosphatase for 1 h in 37°C. All the fragments were further separated via gel electrophoresis using TAE buffer, isolated from gel via Gel/PCR DNA fragments kit (Quiagen) following manufacturer's instruction and proceeded for ligation in the room temperature overnight (reaction mixture in **Tab. 11**).

Table 11: Ligation reaction mixture

Linear vector	20 – 100 ng
Inserted DNA	1:1 – 5:1 (inserted DNA: vector)
10x T4 DNA ligase buffer	2 µl
T4 DNA ligase (5U/µl)	0.5 µl
Deionized water	Up to total volume of 20 µl.

After ligation MTMR10/pcDNA4.0 and MTMR12/pEGFP-N3 vectors were amplified in competent bacteria DH5α with ampicillin and kanamycin (KAN) resistance, respectively, and isolated from bacteria. The presence of fragments in the final vectors was first verified via restriction digestion and finally via sequencing.

6.2.2. Gibson cloning

For preparation of "MTM1/pcDNA4.0" and "MTMR2/pcDNA4.0" constructs Gibson cloning was performed as shown in **Fig. 16**.

Initially, to remove original methylated DNA from amplified MTM1 an MTMR2 PCR products, 1 µl of DpnI (10 U/µl, Fermentas) endonuclease was added for 1.5h at 37°C. pcDNA4.0 vector was amplified (reaction mixture and conditions in **Tab. 9, 10**). PCR products were verified via electrophoresis and then concentrated into 12 µl using "DNA Clean and Concentrator Kits" (Zymo Research, Germany) following manufacturer's instructions.

Working on ice, 100 ng of linearized vector backbone was mixed with desired linearized fragment in ratio 3:2 and refilled by ddH_2O up to 5 µl. This mixture was added into the 15 µl aliquot of Gibson assembly master mix. The whole reaction assembly was incubated at 50°C for 1 h and placed on ice. 5 µl of reaction mixture was then transformed into competent bacteria strand DH5α and growth on agarose plates with AMP resistance. To discover the presence of desired fragments in pcDNA4.0 vectors, vectors were firstly isolated using alkaline lysis mini prep assay and checked via restriction digestion following manufacturer's instructions. Subsequently, plasmids were isolated from the relevant colonies using High-Speed plasmid mini kit and vectors were finally verified via sequencing.

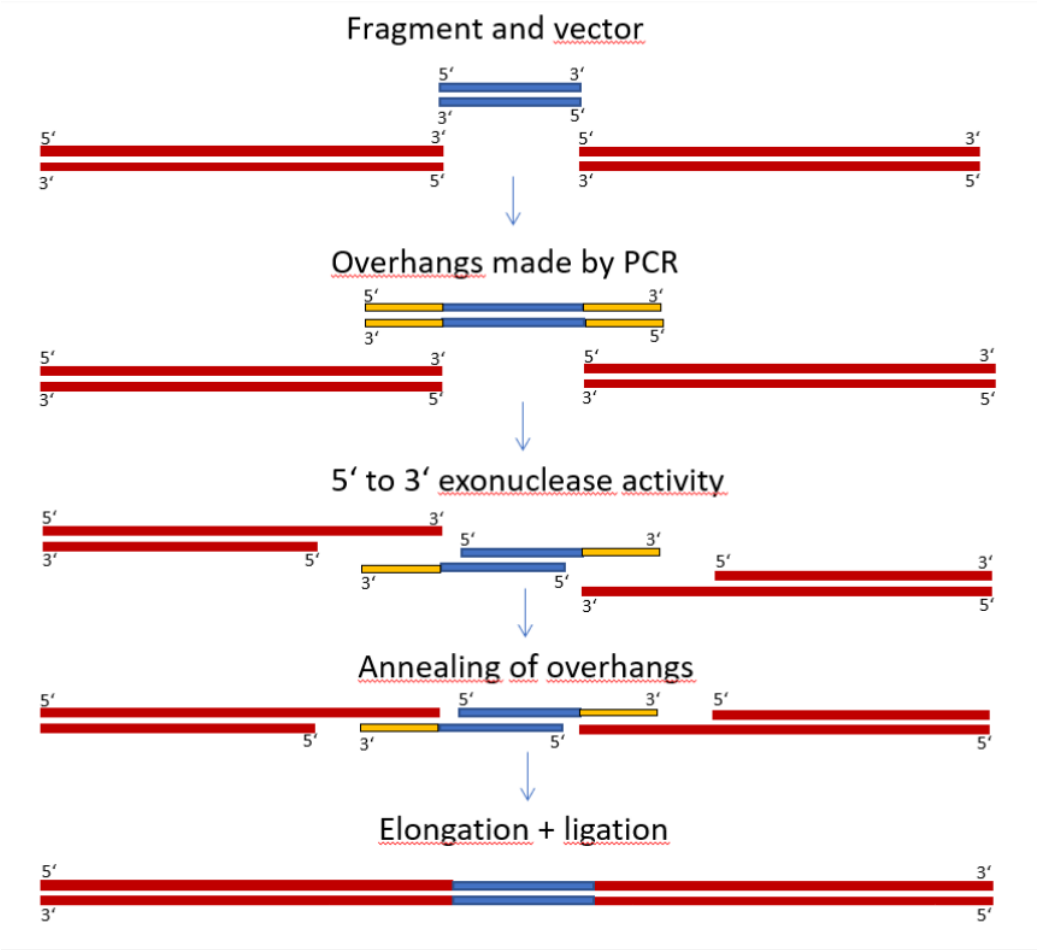

Figure 16: ***Gibson assembly mechanism:*** Gibbson assembly is a molecular cloning method of employing three enzymatic reactions in the following order: 1) 5‘ exonuclease activity 2) 3‘ elongation activity 3) DNA ligase aktivity.

6.2.3. DNA gel electrophoresis

For all the experiments 1% gel was used. Agarose powder was diluted in required volume of TBE buffer. 1/20 000 of ethidium bromide was added to the gel before solidification. DNA

samples with 1/6 of DNA 6x Loading Dye (Thermo Fisher Scientific) were run for 10 V/1 cm of the gel and related to 3 µl of O'GeneRuler 1kb DNA ladder (Fermentas).

6.2.4. Transformation into DH5α

Strain of competent bacteria DH5α was replaced from -80°C on ice. Directly after unmelting, bacteria were mixed with a proper amount of plasmid DNA, incubated on ice for 30 min, followed by heat-shock 42°C/45 s and kept on ice for 2 min. The mixture was incubated with 700 ml of LB buffer 37°C/45 min and centrifuged for 1 min at 14 000 g. After being strained, the pellet was dissolved in the resting LB buffer (approximately 50 µl), spread on agarose plates with appropriate antibiotics and kept overnight in 37°C.

6.2.5. Alkaline lysis mini prep procedure

1.5 ml of inoculated culture was centrifuged 30 s/14000 g. The LB media was decanted and the pellet was re-suspended in 100 of Solution 1 and vortexed. For subsequent lysis 200 µl of Solution 2 was added, gently inverted 10 times and stored for 5 min at room temperature. The mixture was neutralized with 150 µl of Solution 3, gently inverted 10 times and centrifuged for 10 min/14 000 g cooled at 4°C. Immediately after centrifugation, supernatant was carefully transferred into new Eppendorf tube with 950 µl of 100% EtOH and centrifuged for 10 min /14 000 g cooled at 4°C. Afterwards, supernatant was removed and pellet was re-suspended in 400 µl of 70% EtOH and centrifuged 10 min/14 000 g/ 4°C. After thorough removal of all supernatant, the pellet was let dry for 15 min in room temperature and dissolved in 50 µl of ddH_2O.

6.3. PCR mutagenesis

PCR mutagenesis was performed with reaction mixture mentioned in **Tab. 12** according to reaction conditions mentioned in **Tab. 13**.

Table 12: PCR mutagenesis reaction mixture

Template DNA (50 ng/µl)	1 µl
5x HF Buffer	4 µl
F primer (10 µM)	0.5 µl
R primer (10 µM)	0.5 µl
dNTPs (10 mM)	0.5 µl
Phusion polymerase (2 U/µl)	0.25 µl
Deionized water	Up to total volume of 20 µl

Table 13: PCR mutagenesis reaction conditions

Phase:	Temperature:	Time:	Repeats:
1. Primary denaturation	98°C	2 min	
2. Denaturation	98°C	10 s	25 x
3. Annealing	58 - 62°C	20 s	
4. Elongation	72°C	6 min	
5. Final elongation	72°C	5 min	

After PCR reaction, 10 µl of the mixture was first incubated with 1 µl of DpnI in 37°C/1.5 h to eliminate the original methylated template DNA and then transformed into competent bacterial strand DH5α. Resting 10 µl of PCR product mixture was used as a control. Bacteria were seeded on agarose plates with AMP or KAN antibiotics and incubated overnight in 37°C. Selected colonies were inoculated in 3 ml of LB buffer with corresponding antibiotics and let under continuous shaking overnight in 37°C. Plasmids were then isolated from bacteria and verified via sequencing.

6.4. Experiments with human cell lines

6.4.1. Cultivation of human cell lines

Henrietta Lacks (HeLa) cells derived from cervical cancer, Human embryonic kidney 293 (HEK293), RPE-1 and SHS cell lines were cultivated at 37°C in 5% CO_2 atmosphere on plastic dishes with 6 cm in diameter. Before reaching the confluent state, the cells were sub-cultured (2-3 times a week) in laminar flow box. The cells were initially washed from medium with PBS and detached from cultivation dish by 10% trypsin/EDTA (Thermo Fisher Scientific.) After trypsinisation (2-5 min) the activity of an enzyme was blocked by adding of DMEM/FCS. Cells were centrifuged for 4 min/150 g and re-suspended in defined volume of complete DMEM and transferred back to Petri dish.

6.4.2. Freezing cells

Before reaching the fully confluent state, healthy growing cells were passaged as mentioned-. After centrifugation step the pellet was re-suspended in 3 ml of DMEM, 300 µl of 100% DMSO (Sigma Aldrich) was added as a cryoprotectant for the cells and the volume was aliquoted into special cryovials. To achieve gradual freezing for the cells, the cryovials were placed into freezing chambers filled with room tempered isopropanol and stored at -80°C overnight. The frozen cryovials were then moved to the liquid N_2 for proper preservation.

6.4.3. Thawing cells

For the greatest cell viability, the cells were removed from liquid N_2 directly to a pre-warmed water bath. Immediately after unfreezing the cells were transferred into 5 ml of DMEM for dilution of DMSO and centrifuged for 4 min/150 g. The pellet was re-suspended in fresh medium and placed on plastic dish. Cells were further cultivated as described above. At least 2 passages were performed before the cells were proceeded for the experiments.

6.4.4. Seeding and transfection

When prepared for microscopy, HeLa cells and RPE-1 cells were seeded on 12 well plates with coverslips and transiently transfected in an approximate cell density of 30%. When prepared for expression analysis, HEK293 cells were seeded on 12 well plates and transfected in approximately 50% state of confluence. For transfection, either X-treme GENE HP DNA Transfection Reagent (X-treme) (Roche) or polyethylenimine (PEI) jetOPTIMUS (Polyplus) transfection reagents were used (reaction mixture in **Tab. 14**.)

Table 14: Reaction mixture for the transfection with X-treme and PEI reagents

Reagent:	Amount:
Plasmid DNA	1 µg
DMEM buffer	100 µl
Transfection reagent	1.5 µl

When using X-treme transfection reagent, the mixture was kept in room temperature for 20 min, dripped on the cells and incubated in 37°C for 24 h. When using PEI transfection reagent, the mixture was kept in room temperature for 10 min, dripped on the cells and kept in 37°C for 4 hours. Then the medium was substituted with complete DMEM and kept in 37°C for next 20 hours.

6.4.5. Cell starvation

Cells were seeded on 12 well plates with provided coverslips and transiently transfected. 24 hours after transfection, coverslips were washed by PBS and DMEM was substituted for 1 ml of nutrient-free HBSS and incubated under standard conditions for 4 h. Cells then proceeded to classical fixation and permeabilization as mentioned below.

6.4.6. Cell lysis, SDS-PAGE, Western blot assay

Transfected HEK293 cells were washed from growth media and prepared for Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following these steps: working on

ice the cells were lysed with Laemmli buffer with ratio of 100 µl per 1 mil cells, scraped and transferred into Eppendorf tube, centrifuged 30 s/14 000 g, sonicated using ultrasonic processor UP100H (Hielscher) and denaturated in 95 °C for 5 min.

For SDS-PAGE, 9% separating and 3,9% stacking gels were prepared in an electrophoresis cassette. The cassette was placed into an electrophoresis tank and poured by a running buffer. 10-15 µl of the samples were loaded into gel holes with Hamilton syringe needle and the tank was connected to the power supply. The proteins were separated at 120 V and their sizes were related to the PAGE Ruler Plus Pre-stainedProtein Ladder (Thermo Fisher Scientific).

Placed in between two filter papers and soaked with blotting buffer, proteins were transferred from the gel to a nitrocellulose membrane (GE, Healthcare) using semi-dry western blot assay. The assay was optimized at 0.8 mA/cm^2 and run for 45 min on TE77XP (Hoefer) blotting machine. The membrane was then blocked for 45 min in TBST with 5% powdered milk (ROTH) and incubated with primary antibody in 1% milk/TBST overnight in 4 °C. Next day, the membrane was washed 3 times over 30 min by TBST and incubated with secondary antibody (in 1% milk/TBST) with conjugated Horse-Radish Peroxidase (HRP) for 1 h at room temperature. Homemade peroxidase substrates were used to generate signals detected by LAS 4000 (GE, Healthcare).

6.4.7. Fixation and immunostaining

Coverslips with grown cells were washed 3 times by PBS and fixed in 1 ml of MSB/ 3% paraformaldehyde for 10 min. For sub-sequential staining, the cells were washed in PBS and permeabilized by 0.1% Triton X-100 in PBS for 5 min. Cells were then washed 3 times in PBS. The coverslips were stained by primary antibody (diluted in DMEM) and incubated in 4 °C overnight. Next day, the coverslips were washed 3 times in PBS and stained in the same manner with secondary antibody at room temperature for 1 h. Coverslips were again washed 3 times in PBS and mounted into 20 µl of Mowiol solution with Diamidino-2-phenylindole dye (DAPI) (14.3 µM).

6.4.8. Confocal microscopy

If not stated otherwise, the pictures were acquired using z-stack with 200 nm interval between two stacks with a uniform pixel size of 70.8 nm via scanning confocal microscope Leica TSC SP8. Proper excitation and emission filters were set. 63x objective was used along with oil immersion.

All the pictures were processed using Fiji (Schindelin et al. 2012). Images prepared in z-stacks were adjusted to the maximal intensity projection. For co-localization analyses, Pearson's correlation coefficient was computed for each slice of the z-stack of a single image using JACoP plugin (Bolte and Cordelières 2006) The slice with the highest correlation was used as a representative.

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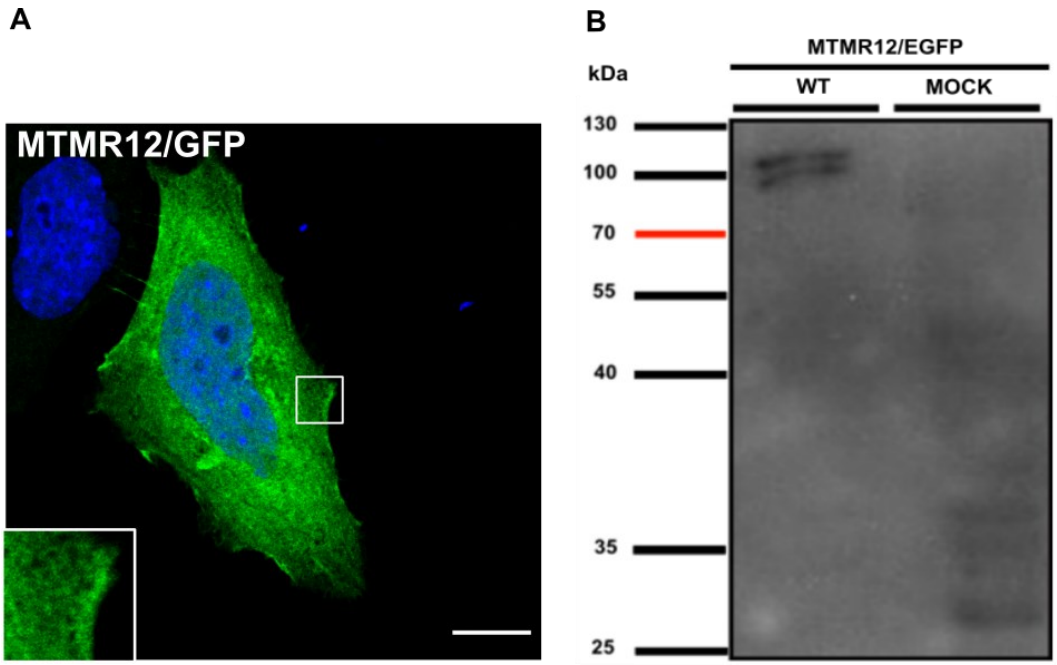
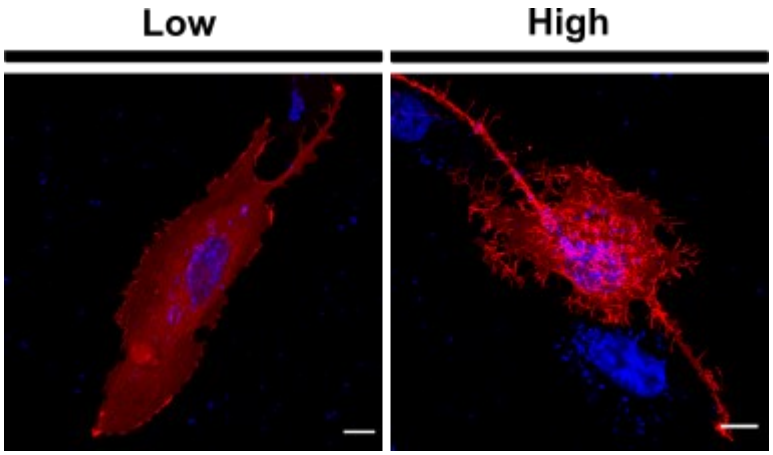
8. Supplementary figures



Supplementary figure 1: ***Overexpressed MTMR12-EGFP is distributed through the cell cytoplasm with occasional presence at the PM.*** **A)** Overexpressed WT MTMR12-EGFP localizes to the cell cytoplasm and to the plasmatic membrane in HeLa cells; Scale bar: 10 μm. **B)** MTMR12-EGFP (113 kDa) is being produced in HEK293 cells. The upper band might stand for MTMR12-EGFP with transient posttranslational modification.



Supplementary figure 2: ***High levels of MTM1-MycHis expression result into formation of extensive PM projections in RPE-1 cells.*** Scale bar: 10 μm.